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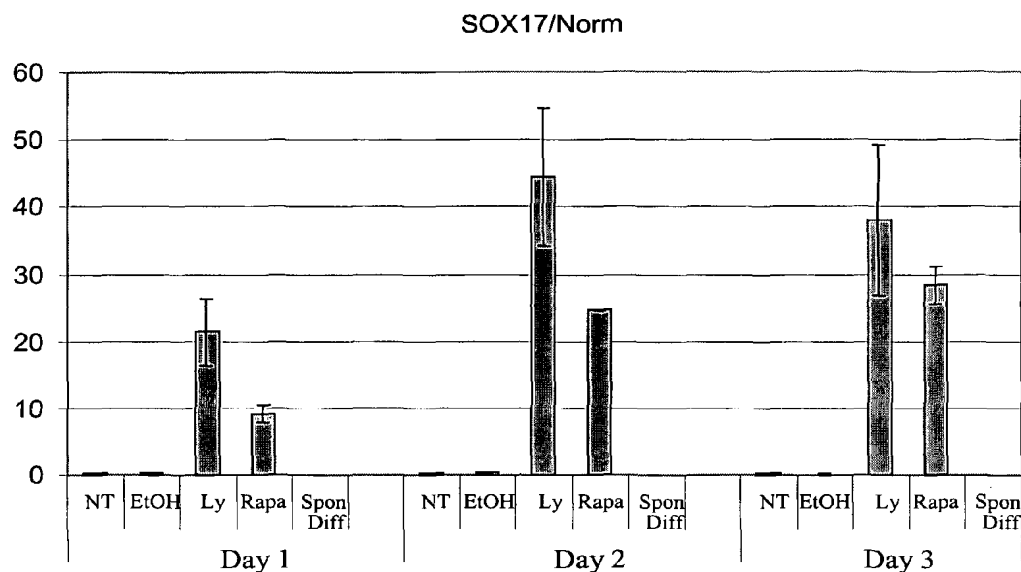
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[Continued on next page]

(54) Title: COMPOSITIONS AND METHODS FOR SELF-RENEWAL AND DIFFERENTIATION IN HUMAN EMBRYONIC STEM CELLS



(57) Abstract: The present invention provides compositions and methods for the production of differentiated mammalian cells. More particularly, the present invention provides cellular differentiation methods employing culturing the cells on a feeder layer or under feeder-free conditions in cell culture and further contacting the cells with an inhibitor of the PI3-kinase pathway and a member of the TGF $\beta$  family for the generation of differentiated mammalian cells from pluripotent mammalian stem cells. Preferably, the differentiated cell is selected from the group consisting of a mesendodermal cell, a mesodermal cell, and an endodermal cell.

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## COMPOSITIONS AND METHODS FOR SELF-RENEWAL AND DIFFERENTIATION IN HUMAN EMBRYONIC STEM CELLS

### 5 BACKGROUND OF THE INVENTION

#### Field of the Invention

[001] The present invention generally relates to compositions and methods for differentiating and culturing pluripotent stem cells, the cells created by these methods and their uses thereof.

10

#### Background Art

[002] Embryonic Stem (ES) cells represent a powerful model system for the investigation of mechanisms underlying pluripotent cell biology and differentiation within the early embryo, as well as providing opportunities for genetic manipulation of mammals and resultant commercial, medical and agricultural applications. Furthermore, appropriate proliferation and differentiation of ES cells can be used to generate an unlimited source of cells suited to transplantation for treatment of diseases that result from cell damage or dysfunction. Other pluripotent cells and cell lines including early primitive ectoderm-like (EPL) cells as described in International Patent Application WO 99/53021, *in vivo* or *in vitro* derived ICM/epiblast, *in vivo* or *in vitro* derived primitive ectoderm, primordial germ cells (EG cells), teratocarcinoma cells (EC cells), and pluripotent cells derived by dedifferentiation or by nuclear transfer will share some or all of these properties and applications.

[003] The successful isolation, long-term clonal maintenance, genetic manipulation and germ-line transmission of pluripotent cells has generally been difficult and the reasons for this are unknown. International Patent Application WO 97/32033 and U.S. Patent No. 5,453,357 describe pluripotent cells including cells from species other than rodents. Human ES cells have been described in International Patent Application WO 00/27995, and in U.S. Patent No. 6200806, and human EG cells have been described in International Patent Application WO 98/43679.

[004] The ability to tightly control differentiation or form homogeneous populations of partially differentiated or terminally differentiated cells by differentiation *in vitro* of pluripotent cells has proved problematic. Current approaches can involve the formation of embryoid bodies from pluripotent cells in a manner that is not controlled

and does not result in homogeneous populations. Mixed cell populations such as those in embryoid bodies of this type are generally unlikely to be suitable for therapeutic or commercial use.

[005] The biochemical mechanisms regulating ES cell pluripotency and differentiation are very poorly understood. However, the limited empirical data available (and much anecdotal evidence) suggests that the continued maintenance of pluripotent ES cells under *in vitro* culture conditions is dependent upon the presence of cytokines and growth factors present in the extracellular serum milieu. A number of such factors such as insulin, IGF(s) and FGF(s) have been found to activate intracellular signaling events through the lipid kinase phosphatidylinositol 3-kinase (PI3-kinase) (Carpenter & Cantley, (1996) Curr. Opin. Cell. Biol., 8: 153-158). In response to the binding of these soluble factors to specific cell surface receptors, PI3-kinase is recruited to the intracellular membrane surface where it initiates a cascade of secondary signaling events leading to the functional regulation of several downstream intracellular targets that influence diverse biological processes. Amongst the downstream targets of PI3-kinase is the protein kinase called 'mammalian Target Of Rapamycin' (mTOR). Stimulation of mTOR both precedes and is necessary for activation of ribosomal p70 S6 kinase, a serine/threonine kinase that is pivotal to the regulation of the protein synthetic machinery (Chung et al., (1994) Nature, 370: 71-75).

[006] There is a need, therefore, to identify methods and compositions for the production of a population of cells enriched in a cell lineage through the manipulation of the PI3-kinase signaling pathway, the maintenance or stabilization, and proliferation of these cells, and the products of their further differentiation.

## 25 SUMMARY OF THE INVENTION

[007] It is an object of the present invention to overcome, or at least alleviate, one or more of the difficulties or deficiencies associated with the prior art. One embodiment of the present invention relates to novel, defined processes for the production of definitive endoderm cells in culture using pluripotent cells. These processes provide the basis for efficient production of endodermal derived tissues such as pancreas, liver, lung, stomach, intestine and thyroid.

[008] The present invention contemplates a composition comprising a population of isolated differentiated mammalian cells, wherein the cells are differentiated from a pluripotent cell *in vitro*, and wherein greater than approximately

50% of the cells express SOX17 but do not express AFP. In one embodiment of the invention, greater than approximately 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% of the cells express SOX17 but do not express AFP.

5 [009] The invention further contemplates a composition comprising a homogenous population of isolated definitive endoderm cells, wherein the cells were differentiated in an *in vitro* culture, and wherein greater than approximately 50% of the population are definitive endoderm cells. In certain embodiments, greater than approximately 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%,  
10 96%, 97%, 98%, or 99% of the population are definitive endoderm cells. In one embodiment, the definitive endoderm cells express SOX17 but do not express AFP.

[010] In an additional embodiment of the invention, the population has increased expression of HNF4alpha, GATA4, Mix1, and Msx1, and decreased expression of AFP in comparison to a population of spontaneously differentiating  
15 pluripotent cells. In a further embodiment, the population has increased expression of goosecoid, Brachyury, and Cerebrus, and decreased expression of AFP in comparison to a population of spontaneously differentiating pluripotent cells. It is also contemplated that the population can have increased expression of MIX1, goosecoid, and Cerebrus, and decreased expression of AFP in comparison to a population of  
20 spontaneously differentiating pluripotent cells. In one embodiment, the population does not have increased expression of SOX1 in comparison to a population of spontaneously differentiating pluripotent cells. In another embodiment, the population does not have increased expression of SOX7 in comparison to a population of spontaneously differentiating pluripotent cells. In another embodiment, the cells  
25 display similarly low expression of thrombomodulin as seen in a population of pluripotent cells as determined, for example, by flow cytometry.

[011] The invention further encompasses a method of differentiating a pluripotent mammalian cell comprising: (a) providing the pluripotent mammalian cell, and (b) contacting the pluripotent mammalian cell with an effective amount of an  
30 inhibitor of the PI3-kinase signaling pathway and a member of the TGFβ family to at least partially differentiate the pluripotent cell to a cell of the endoderm lineage. In one embodiment, the member of the TGFβ family is selected from the group consisting of Nodal, Activin A, Activin B, TGF-β, BMP2, BMP4, and mixtures of the foregoing. In

certain embodiments, the member of the TGF $\beta$  family is Activin A. It is contemplated that the member of the TGF $\beta$  can be exogenously added to the pluripotent mammalian cell in a substantially pure form, or can be present in a conditioned medium, as a substance produced by the feeder layer.

5 [012] In one embodiment, the differentiated cell is isolated after step (b).

[013] It is contemplated that the pluripotent cells have been differentiated by contact with a PI3-kinase inhibitor and a member of the TGF $\beta$  family.

[014] In one embodiment, the cells are dissociated to an essentially single cell culture prior to being contacted with the inhibitor and the member of the TGF $\beta$  family.

10 The cells can be dissociated using a protease, such as, but not limited to, trypsin.

[015] In one embodiment, the cells are contacted with the PI3-kinase inhibitor and the member of the TGF $\beta$  family after being plated for between approximately 12 hours to approximately 6 days, after being plated for between approximately 12 hours to approximately 48 hours, or after being plated for approximately 24 hours. In certain  
15 embodiments, the pluripotent cells are plated at a concentration of less than approximately  $2.5 \times 10^4$  cells/35 mm dish, of at least approximately  $2.5 \times 10^4$  cells/35 mm dish, between approximately  $2.5 \times 10^4$  to approximately  $2 \times 10^5$  cells/35 mm dish, between approximately  $5 \times 10^4$  to approximately  $2 \times 10^5$  cells/35 mm dish, of less than approximately  $4 \times 10^5$  cells/35 mm dish, or at a density of greater than  $4 \times 10^5$  cells/35  
20 mm dish.

[016] In one embodiment, the cells are contacted with the PI3-kinase inhibitor and the member of the TGF $\beta$  family for greater than approximately 24 hours, for greater than approximately 48 hours, for greater than approximately 72 hours, or for approximately 72 hours. It is preferred that a composition comprising the inhibitor of  
25 the PI3-kinase pathway and the member of the TGF $\beta$  family is effective in causing differentiation of a pluripotent mammalian cell towards an endodermal lineage after the cell has been cultured with the composition for greater than approximately 24 hours. It is also contemplated that a composition comprising the inhibitor of the PI3-kinase pathway and the member of the TGF $\beta$  family is effective in causing differentiation of a  
30 pluripotent mammalian cell towards an endodermal lineage when the cell has been plated for greater than approximately 12 hours before it is contacted with the composition, or when the cell has been plated for approximately 24 hours before it is contacted with the composition.

[017] The present invention further encompasses a composition for culturing cells, comprising a cell culture medium, an inhibitor of the PI3-kinase pathway, and a member of the TGF $\beta$  family. In certain embodiments of the foregoing, the inhibitor is selected from the group consisting of LY 294002, Rapamycin, wortmannin, lithium chloride, Akt inhibitor I, Akt inhibitor II, Akt inhibitor III, NL-71-101, and mixtures of the foregoing. In one embodiment, the inhibitor is Rapamycin. In certain embodiments, Rapamycin is initially present at a concentration of approximately 0.1 nM to approximately 500 nM, approximately 0.5 nM to approximately 250 nM, approximately 1.0 nM to approximately 150 nM, or approximately 1.5 nM to approximately 30 nM. In another embodiment, the inhibitor is LY 294002. In certain embodiments, LY 294002 is initially present at a concentration of approximately 1  $\mu$ M to approximately 500  $\mu$ M, approximately 2.5  $\mu$ M to approximately 400  $\mu$ M, approximately 5  $\mu$ M to approximately 250  $\mu$ M, approximately 10  $\mu$ M to approximately 200  $\mu$ M or approximately 20  $\mu$ M to approximately 163  $\mu$ M. In another embodiment, the inhibitor is AktI-II. In certain embodiments, AktI-II is initially present at a concentration of approximately 0.1  $\mu$ M to approximately 500  $\mu$ M, approximately 1  $\mu$ M to approximately 250  $\mu$ M, approximately 5  $\mu$ M to approximately 20  $\mu$ M, approximately 10  $\mu$ M to approximately 100  $\mu$ M or approximately 40  $\mu$ M.

[018] In a further embodiment, contacting the pluripotent cell with the inhibitor of the PI3-kinase pathway activates GSK3.

#### BRIEF DESCRIPTION OF THE DRAWINGS

[019] Figures 1A-D are photomicrographs at 10X magnification, showing the morphology of the human BG01 cell line. (A) shows untreated BG01 cells, (B) shows BG01 cells treated with 80  $\mu$ M LY 294002, (C) shows BG01 cells treated with 30 nM rapamycin; and (D) shows the spontaneous differentiation of BG01 cells.

[020] Figures 2A-D are photomicrographs at 20X magnification, showing the morphology of the human BG01 cell line. (A) shows untreated BG01 cells, (B) shows BG01 cells treated with 80  $\mu$ M LY 294002, (C) shows BG01 cells treated with 30 nM rapamycin; and (D) shows the spontaneous differentiation of BG01 cells.

[021] Figures 3A-E show the flow cytometry analysis of CD9 expression in human BG01 cells. (A) shows the secondary antibody alone, (B) shows untreated cells, (C) shows cells treated with 80  $\mu$ M LY 294002, (D) shows cells treated with 30 nM

rapamycin, and (E) shows spontaneously differentiated cells. Figures 3F-I show flow cytometry analysis of CD9 expression during embryoid body differentiation. (F) shows the secondary antibody alone, (G) shows undifferentiated BG01 cells, (H) shows embryoid bodies at day 3, and (I) shows embryoid bodies at day 5.

5 [022] Figure 4A shows RT-PCR analysis of lineage markers in BG01 cells, comparing expression differences between LY 294002 (80  $\mu$ M) treatment and the spontaneous differentiation of the cells. Figure 4B shows RT-PCR analysis of lineage markers in BG01 cells, comparing expression differences between LY294002 (80  $\mu$ M) and rapamycin (30 nM) treatments.

10 [023] Figures 5A-G demonstrate that treatment of hES cell line BG01 with either LY 294002 or Rapamycin quantitatively induces the expression of genes strongly associated with mesendoderm, however, LY294002 or Rapamycin treatment do not induce expression of the pan-Neurectodermal marker SOX1, or the pan-Extraembryonic marker, SOX7. "NT" indicates untreated cells; "ETOH" indicates  
 15 vehicle control; "Ly" indicates treatment with 80  $\mu$ M LY 294002; "RAPA" indicates treatment with 30 nM Rapamycin; "Spon Diff" indicates spontaneous differentiation of hES cells. The figures show the relative gene expression (Y axis) as a function of treatment and time (X axis). Figure 5A shows relative SOX17 gene expression; Figure 5B shows relative Mix1 gene expression; Figure 5C shows relative goosecoid (GSC)  
 20 gene expression; Figure 5D shows relative GATA4 expression; Figure 5E shows relative Cerebrus expression; Figure 5F shows relative nodal gene expression; Figure 5A shows relative Brachyury gene expression; Figure 5H shows relative SOX7 gene expression; and Figure 5I shows relative SOX1 gene expression.

[024] Figure 6 shows flow cytometry analysis of thrombomodulin expression  
 25 in human BG01 cells. The gray histogram shows untreated cells, while the black histogram shows cells treated with 80  $\mu$ M LY 294002.

[025] Figures 7A-T demonstrate that treatment of hES cell lines BG02 and H1 with LY 294002 induces the expression of genes strongly associated with mesendoderm. NT" indicates untreated cells; "LY" indicates treatment with 80  $\mu$ M LY  
 30 294002. Expression levels were examined by Q-PCR after approximately 72 hours in culture with LY 294002. Figures 7A and K show relative AFP expression; Figures 7B and L show relative Bry expression; Figures 7C and M show relative FoxA2 expression; Figures 7D and N show relative GSC expression; Figures 7E and O show



relative Mox1 expression; Figures 7F and P show relative MixL1 expression; Figures 7G and Q show relative Sox1 expression; Figures 7H and R show relative Sox 17 expression; Figures 7I and S show relative THBD expression; and Figures 7J and T show relative ZIC1 expression. Assays were performed in triplicate and are shown as  
5 +/- SEM.

[026] Figure 8A shows that MEF-CM or Activin/Nodal are necessary for LY 294002-dependent hESC-DE formation. (A) shows Sox17 mRNA levels (fold-increase over untreated) as evaluated by Q-PCR under various conditions for 4 days; LY 294002 (60  $\mu$ M), Activin A (100 ng/ml), Nodal (1  $\mu$ g/ml). UCM indicates unconditioned  
10 media. Figure 8B shows LY 294002, MEF-CM dependent Sox17 expression is suppressed by inhibitors of Activin signaling but not suppressed by inhibitors of BMP or FGF signaling. Sox 17 expression was evaluated by Q-PCR under culture conditions competent to support hESC-DE formation in the presence or absence of Chordin (500 ng/ml), Follistatin (500 ng/ml), Lefty-A (500 ng/ml), Noggin (500 ng/ml), SB-43152  
15 (10  $\mu$ M), or SU-5402 (5  $\mu$ M). The first column of Figure 8B shows untreated cells. Assays were performed in triplicate and are shown as +/- SEM.

[027] Figure 9 shows Q-PCR results indicating that addition of approximately 40  $\mu$ M of the AKT inhibitor AKT1-II can reproduce the effects of LY 294002 in promoting DE formation.

20 [028] Figure 10 shows that activation of GSK3 is required for LY 294002 to promote DE. Addition of the GSK3 inhibitor, BIO, but not MeBio, blocks LY 294002 induced DE formation. DE formation is indicated by Q-PCR analysis of the cells, showing Sox17 mRNA levels as a fold-increase over untreated cells. Assays were performed in triplicate and are shown as +/- SEM.

25 [029] Figures 11A and B show that knockdown of GSK3 expression with two specific RNAi molecules, but not a mutant RNAi, blocks the ability of LY 294002 to promote DE formation. Cells were assayed by GSK3 and Sox17 immunocytochemistry at the times indicated.

[030] Figure 12A shows a control kidney that does not contain an implant  
30 (left) and a kidney that contains an implant of hESCs treated for 4 days with 60  $\mu$ M LY 294002 (right). The implant was grown under the kidney capsule of 5-week-old male SCID-beige mice for 6 weeks. The LY 294002 treated HESCs formed a large mass on the kidney. Figures 12B-G show photomicrographs of immunostainings of LY 294002

treated aggregates after culture under a kidney capsule for approximately 6 weeks. The aggregates express TTF-1 (B), AFP (C), villin (D), gastrin (E), HSA (F) and LFABP (G), indicating that the LY 294002 treated cells differentiate to derivatives of endoderm. Figures 12H and 12I demonstrate Q-PCR data showing a 1000-fold increase in FABP1 mRNA and a 4500-fold increase in albumin mRNA in LY294002 treated HESCs relative to LY 294002 treated cells that were not implanted. Assays were performed in triplicate and are shown as +/- SEM.

#### DETAILED DESCRIPTION OF THE INVENTION

10 [031] Applicant has demonstrated that culturing pluripotent mammalian cells with an inhibitor of PI3-kinase and a member of the TGF $\beta$  family generates differentiated cells wherein the cells have greater homogeneity than spontaneously differentiated cells.

15 [032] The present invention contemplates a composition comprising a population of isolated differentiated mammalian cells, wherein the cells are differentiated from a pluripotent cell *in vitro*, and wherein greater than approximately 50% of the cells express SOX17 but do not express AFP. In one embodiment of the invention, greater than approximately 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% of the cells express SOX17 but do not  
20 express AFP.

[033] The invention further contemplates a composition comprising a homogenous population of isolated definitive endoderm cells, wherein the cells were differentiated in an *in vitro* culture, and wherein greater than approximately 50% of the population are definitive endoderm cells. In certain embodiments, greater than  
25 approximately 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% of the population are definitive endoderm cells. In one embodiment, the definitive endoderm cells express SOX17 but do not express AFP.

[034] In an additional embodiment of the invention, the population has increased expression of HNF4 $\alpha$ , GATA4, Mix1, and Msx1, and decreased  
30 expression of AFP in comparison to a population of spontaneously differentiating pluripotent cells. In a further embodiment, the population has increased expression of Goosecoid, Brachyury, and Cerebrus, and decreased expression of AFP in comparison to a population of spontaneously differentiating pluripotent cells. It is also contemplated that the population can have increased expression of MIX1, Goosecoid,

and Cerebrus, and decreased expression of AFP in comparison to a population of spontaneously differentiating pluripotent cells. In one embodiment, the population does not have increased expression of SOX1 in comparison to a population of spontaneously differentiating pluripotent cells. In another embodiment, the population does not have increased expression of SOX7 in comparison to a population of spontaneously differentiating pluripotent cells. In another embodiment, the cells display similarly low expression of thrombomodulin as seen in a population of pluripotent cells as determined, for example, by flow cytometry.

[035] The invention further encompasses a method of differentiating a pluripotent mammalian cell comprising: (a) providing the pluripotent mammalian cell, and (b) contacting the pluripotent mammalian cell with an effective amount of an inhibitor of the PI3-kinase signaling pathway and a member of the TGF $\beta$  family to at least partially differentiate the pluripotent cell to a cell of the endoderm lineage. In one embodiment, the differentiated cell is isolated after step (b).

[036] It is contemplated that the pluripotent cells have been differentiated by contact with a composition comprising a PI3-kinase inhibitor and a member of the TGF $\beta$  family. In one embodiment, the cells are dissociated to an essentially single cell culture prior to being contacted with the composition. The cells can be dissociated using a protease, such as, but not limited to, trypsin. The PI3-kinase inhibitor and the member of the TGF $\beta$  family do not need to be added simultaneously to the cell or cell culture, however, it is contemplated that during a point in culture, both the PI3-kinase inhibitor and the member of the TGF $\beta$  family will both be present in the composition.

[037] In one embodiment, the cells are contacted with the composition after being plated for between approximately 12 hours to approximately 6 days, after being plated for between approximately 12 hours to approximately 48 hours, or after being plated for approximately 24 hours. In one embodiment, the cells are contacted with the composition for greater than approximately 24 hours, for greater than approximately 48 hours, for greater than approximately 72 hours, or for approximately 72 hours. It is preferred that the composition is effective in causing differentiation of a pluripotent mammalian cell towards an endodermal lineage after the cell has been cultured with the composition for greater than approximately 24 hours. It is also contemplated that the composition is effective in causing differentiation of a pluripotent mammalian cell towards an endodermal lineage when the cell has been plated for greater than

approximately 12 hours before it is contacted with the composition, or when the cell has been plated for approximately 24 hours before it is contacted with the composition.

[038] In certain embodiments, the pluripotent cells are plated at a concentration of less than approximately  $2.5 \times 10^4$  cells/35 mm dish, of at least  
5 approximately  $2.5 \times 10^4$  cells/35 mm dish, between approximately  $2.5 \times 10^4$  to approximately  $2 \times 10^5$  cells/35 mm dish, between approximately  $5 \times 10^4$  to approximately  $2 \times 10^5$  cells/35 mm dish, of less than approximately  $4 \times 10^5$  cells/35 mm dish, or at a density of greater than  $4 \times 10^5$  cells/35 mm dish.

[039] The present invention further encompasses a composition for culturing  
10 cells, comprising a cell culture medium, an inhibitor of the PI3-kinase pathway, and a member of the TGF $\beta$  family. It is contemplated that the member of the TGF $\beta$  can be exogenously added to the pluripotent mammalian cell in a substantially pure form, or can be present in a conditioned medium, as a substance produced by the feeder layer.

[040] In certain embodiments of the invention, the inhibitor is selected from  
15 the group consisting of LY 294002, Rapamycin, wortmannin, lithium chloride, Akt inhibitor I, Akt inhibitor II, Akt inhibitor III, NL-71-101, and mixtures of the foregoing. In one embodiment, the inhibitor is Rapamycin. In certain embodiments, Rapamycin is initially present at a concentration of approximately 0.1 nM to approximately 500 nM, approximately 0.5 nM to approximately 250 nM, approximately 1.0 nM to  
20 approximately 150 nM, or approximately 1.5 nM to approximately 30 nM. In another embodiment, the inhibitor is LY 294002. In certain embodiments, LY 294002 is initially present at a concentration of approximately 1  $\mu$ M to approximately 500  $\mu$ M, approximately 2.5  $\mu$ M to approximately 400  $\mu$ M, approximately 5  $\mu$ M to approximately 250  $\mu$ M, approximately 10  $\mu$ M to approximately 200  $\mu$ M or approximately 20  $\mu$ M to  
25 approximately 163  $\mu$ M. In another embodiment, the inhibitor is AktI-II. In certain embodiments, AktI-II is initially present at a concentration of approximately 0.1  $\mu$ M to approximately 500  $\mu$ M, approximately 1  $\mu$ M to approximately 250  $\mu$ M, approximately 5  $\mu$ M to approximately 20  $\mu$ M, approximately 10  $\mu$ M to approximately 100  $\mu$ M, or approximately 40  $\mu$ M.

30 [041] The cell culture composition can further comprise an FGF. In one embodiment, the FGF is bFGF. bFGF is initially present at a concentration of approximately 0.1 ng/ml to approximately 100 ng/ml, approximately 0.5 ng/ml to approximately 50 ng/ml, approximately 1 ng/ml to approximately 25 ng/ml,

approximately 1 ng/ml to approximately 12 ng/ml, or is initially present at a concentration of approximately 8 ng/ml.

[042] In a further embodiment, the cell culture medium is a conditioned medium. The conditioned medium can be obtained from a feeder layer. It is contemplated that the feeder layer comprises fibroblasts, and in one embodiment, comprises embryonic fibroblasts. In a further embodiment, the conditioned medium comprises DMEM/F-12 (50/50), approximately 20% KSR, approximately 0.1 mM NEAA, approximately 2 mM L-Glutamine, approximately 50 U/ml penicillin, approximately 50 µg/ml streptomycin, and approximately 8 ng/ml bFGF. In other embodiments, the concentration of KSR can be adjusted, or can be substituted with serum. In one embodiment, KSR is present in the conditioned medium at a concentration of approximately 2%.

[043] It is contemplated that the methods and compositions of the invention comprise a member of the TGFβ family. In certain embodiments, the member of the TGFβ family is selected from the group consisting of Nodal, Activin A, Activin B, TGF-β, BMP2, BMP4, and mixtures of the foregoing. In certain embodiments, the member of the TGF-β family is Activin A or Nodal. It is contemplated that Activin A is initially present at a concentration of approximately 1 ng/ml to approximately 1 mg/ml, approximately 10 ng/ml to approximately 500 ng/ml, approximately 25 ng/ml to approximately 250 ng/ml, approximately 50 ng/ml to approximately 200 ng/ml, or approximately 100 ng/ml. In other embodiments, Nodal is initially present at a concentration of approximately 10 ng/ml to approximately 250 µg/ml, approximately 50 ng/ml to approximately 125 µg/ml, approximately 100 ng/ml to approximately 50 µg/ml, approximately 500 ng/ml to approximately 25 µg/ml, approximately 0.5 µg/ml to approximately 5 µg/ml or approximately 1 µg/ml. [

[044] In one embodiment, contacting the pluripotent cell with the inhibitor of the PI3-kinase pathway activates GSK3.

[045] Unless otherwise noted, the terms used herein are to be understood according to conventional usage by those of ordinary skill in the relevant art. In addition to the definitions of terms provided below, definitions of common terms in molecular biology may also be found in Rieger *et al.*, 1991 Glossary of genetics: classical and molecular, 5th Ed., Berlin: Springer-Verlag; and in Current Protocols in Molecular Biology, F.M. Ausubel *et al.*, Eds., Current Protocols, a joint venture

between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (1998 Supplement). It is to be understood that as used in the specification and in the claims, "a" or "an" can mean one or more, depending upon the context in which it is used. Thus, for example, reference to "a cell" can mean that at least one cell can be utilized.

5    **[046]**           The present invention may be understood more readily by reference to the following detailed description of the preferred embodiments of the invention and the Examples included herein. However, before the present compositions and methods are disclosed and described, it is to be understood that this invention is not limited to specific nucleic acids, specific polypeptides, specific cell types, specific host cells,  
10   specific conditions, or specific methods, etc., as such may, of course, vary, and the numerous modifications and variations therein will be apparent to those skilled in the art.

**[047]**           Standard techniques for cloning, DNA isolation, amplification and purification, for enzymatic reactions involving DNA ligase, DNA polymerase,  
15   restriction endonucleases and the like, and various separation techniques are those known and commonly employed by those skilled in the art. A number of standard techniques are described in Sambrook *et al.*, 1989 Molecular Cloning, Second Edition, Cold Spring Harbor Laboratory, Plainview, New York; Maniatis *et al.*, 1982 Molecular Cloning, Cold Spring Harbor Laboratory, Plainview, New York; Wu (Ed.) 1993 Meth.  
20   Enzymol. 218, Part I; Wu (Ed.) 1979 Meth. Enzymol. 68; Wu et al., (Eds.) 1983 Meth. Enzymol. 100 and 101; Grossman and Moldave (Eds.) 1980 Meth. Enzymol. 65; Miller (ed.) 1972 Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York; Old and Primrose, 1981 Principles of Gene Manipulation, University of California Press, Berkeley; Schleif and Wensink, 1982 Practical Methods  
25   in Molecular Biology; Glover (Ed.) 1985 DNA Cloning Vol. I and II, IRL Press, Oxford, UK; Hames and Higgins (Eds.) 1985 Nucleic Acid Hybridization, IRL Press, Oxford, UK; and Setlow and Hollaender 1979 Genetic Engineering: Principles and Methods, Vols. 1-4, Plenum Press, New York. Abbreviations and nomenclature, where employed, are deemed standard in the field and commonly used in professional journals  
30   such as those cited herein.

**[048]**           Human pluripotent cells offer unique opportunities for investigating early stages of human development as well as for therapeutic intervention in several disease states, such as diabetes mellitus and Parkinson's disease. For example, the use of insulin-producing  $\beta$ -cells derived from hESCs would offer a vast improvement over

current cell therapy procedures that utilize cells from donor pancreases. Currently cell therapy treatments for diabetes mellitus, which utilize cells from donor pancreases, are limited by the scarcity of high quality islet cells needed for transplant. Cell therapy for a single Type I diabetic patient requires a transplant of approximately  $8 \times 10^8$  pancreatic islet cells (Shapiro *et al.*, 2000, N Engl J Med 343:230-238; Shapiro *et al.*, 2001a, Best Pract Res Clin Endocrinol Metab 15:241-264; Shapiro *et al.*, 2001b, Bmj 322:861). As such, at least two healthy donor organs are required for to obtain sufficient islet cells for a successful transplant. HESCs offer a source of starting material from which to develop substantial quantities of high quality differentiated cells for human cell therapies.

[049] As used herein, the terms “biologically active component” or “bioactive component” and “bioactive factor” refer to any compound or molecule that induces a pluripotent cell to partially or terminally differentiate, wherein said differentiation is due at least in part to inhibition of signaling through the PI3-kinase pathway. While the bioactive component may be as described below, the term is not limited thereto. The term “bioactive component” as used herein includes within its scope a natural or synthetic molecule or molecules which exhibit(s) similar biological activity.

[050] As used herein, the term “inhibitor of the PI3-kinase pathway” refers to any molecule or compound that decreases the activity of PI3-kinase or at least one molecule downstream of PI3-kinase in a cell contacted with the inhibitor. The invention encompasses, e.g., PI3-kinase antagonists, antagonists of the PI3-kinase signal transduction cascade, compounds that decrease the synthesis or expression of endogenous PI3-kinase, compounds that decrease release of endogenous PI3-kinase, and compounds that inhibit activators of PI3-kinase activity. In certain embodiments of the foregoing, the inhibitor is selected from the group consisting of Rapamycin, LY 294002, wortmannin, lithium chloride, Akt inhibitor I, Akt inhibitor II (SH-5), Akt inhibitor III (SH-6), NL-71-101, and mixtures of the foregoing. Akt inhibitor I, II, Akt III, and NL-71-101 are commercially available from Calbiochem. In other embodiments, the inhibitor is selected from the group consisting of Rapamycin and LY 294002. In a further embodiment, the inhibitor comprises LY 294002. In another embodiment, the inhibitor comprises AktI-II. In other embodiments, the inhibitor is a molecule that inhibits an upstream component of the PI3-kinase signaling pathway. In particular embodiments of the foregoing, the inhibitor is an inhibitor of an IGF or FGF

receptor. It is understood that combinations of inhibitors may be used to elicit the desired effect.

[051] In one embodiment, the pluripotent cells are contacted with an effective amount of the inhibitor of the PI3-kinase pathway. As used herein, the term "effective amount" refers to that concentration of inhibitor that is sufficient to decrease the activity of PI3-kinase or at least one molecule downstream of PI3-kinase in a cell contacted with the inhibitor and a member of the TGF $\beta$  family to effect differentiation of a pluripotent cell towards mesendoderm, and preferably towards endoderm. Alternatively, it refers to that concentration of activator that is sufficient to increase the activity of PI3-kinase or at least one molecule downstream of PI3-kinase in a cell contacted with the activator.

[052] As used herein when referring to a cell, cell line, cell culture or population of cells, the term "isolated" refers to being substantially separated from the natural source of the cells such that the cell, cell line, cell culture, or population of cells are capable of being cultured *in vitro*. In addition, the term "isolating" is used to refer to the physical selection of one or more cells out of a group of two or more cells, wherein the cells are selected based on cell morphology and/or the expression of various markers.

[053] As used herein, the term "express" refers to the transcription of a polynucleotide or translation of a polypeptide in a cell, such that levels of the molecule are measurably higher in a cell that expresses the molecule than they are in a cell that does not express the molecule. Methods to measure the expression of a molecule are well known to those of ordinary skill in the art, and include without limitation, Northern blotting, RT-PCT, *in situ* hybridization, Western blotting, and immunostaining.

[054] As used herein, the term "contacting" (i.e., contacting a cell e.g. a pluripotent cell, with an compound) is intended to include incubating the compound and the cell together *in vitro* (e.g., adding the compound to cells in culture). The term "contacting" is not intended to include the *in vivo* exposure of cells to an inhibitor of the PI3-kinase pathway and the member of the TGF $\beta$  family that may occur naturally in a subject (i.e., exposure that may occur as a result of a natural physiological process). The step of contacting the cell with an inhibitor of the PI3-kinase pathway and the member of the TGF $\beta$  family can be conducted in any suitable manner. For example, the cells may be treated in adherent culture, or in suspension culture. It is understood



that the cells contacted with the inhibitor of the PI3-kinase pathway and the member of the TGF $\beta$  family may be further treated with other cell differentiation environments to stabilize the cells, or to differentiate the cells further.

[055] In one embodiment of the invention, the cells comprise an isolated  
5 nucleic acid molecule whose expression modulates signaling of the PI3-kinase pathway. In accordance with the present invention, a nucleic acid molecule can be transformed into an embryonic cell population of the present invention to inhibit or activate particular genes or gene products, thereby modulating differentiation of the cells. In one embodiment of the foregoing, the cell is an embryonic stem cell that comprises an  
10 isolated nucleic acid molecule encoding a dominant negative constitutively active form of a protein that is part of the PI3-kinase signaling pathway.

[056] The compositions and methods described herein have several useful features. For example, the compositions and methods described herein are useful for modeling the early stages of human development. Furthermore, the compositions and  
15 methods described herein can also serve for therapeutic intervention in disease states, such as diabetes mellitus. For example, since definitive endoderm serves as the source for only a limited number of tissues, it can be used in the development of pure tissue or cell types.

[057] A crucial stage in early human development termed gastrulation occurs  
20 2-3 weeks after fertilization. Gastrulation is extremely significant because it is at this time that the three primary germ layers are first specified and organized (Lu *et al.*, 2001 Curr Opin Genet Dev 11:384-392; Schoenwolf & Smith, 2000 Methods Mol Biol 135:113-125). The ectoderm is responsible for the eventual formation of the outer coverings of the body and the entire nervous system whereas the heart, blood, bone,  
25 skeletal muscle and other connective tissues are derived from the mesoderm. Definitive endoderm is defined as the germ layer that is responsible for formation of the entire gut tube which includes the esophagus, stomach and small and large intestines, and the organs which derive from the gut tube such as the lungs, liver, thymus, parathyroid and thyroid glands, gall bladder and pancreas (Grapin-Botton & Melton, 2000 Trends Genet  
30 16:124-130; Kimelman & Griffin, 2000, Curr Opin Genet Dev 10:350-356; Tremblay et al., 2000 Development 127:3079-3090; Wells & Melton, 1999 Annu Rev Cell Dev Biol 15:393-410; Wells & Melton, 2000 Development 127:1563-1572). A very important distinction should be made between the definitive endoderm and the completely separate lineage of cells termed primitive endoderm. The primitive

endoderm is primarily responsible for formation of extra-embryonic tissues, mainly the parietal and visceral endoderm portions of the placental yolk sac and the extracellular matrix material of Reichert's membrane.

[058] During gastrulation, the process of definitive endoderm formation begins with a cellular migration event in which mesendoderm cells (cells competent to form mesoderm or endoderm) migrate through a structure called the primitive streak. Definitive endoderm is derived from cells, which migrate through the anterior portion of the streak and through the node (a specialized structure at the anterior-most region of the streak). As migration occurs, definitive endoderm populates first the most anterior gut tube and culminates with the formation of the posterior end of the gut tube.

[059] *In vivo* analyses of the formation of definitive endoderm, such as the studies in Zebrafish and *Xenopus* (Conlon et al., 1994 Development 120:1919-1928; Feldman et al., 1998 Nature 395:181-185; Zhou et al., 1993 Nature 361:543-547; Aoki et al., 2002 Dev Biol 241:273-288; Dougan et al., 2003 Development 130:1837-1851; Tremblay et al., 2000 Development 127:3079-3090; Vincent et al., 2003 Genes Dev 17:1646-1662; Alexander et al., 1999 Dev. Biol. 215:343-357; Alexander & Stainier, 1999 Curr Biol 9:1147-1157; Kikuchi et al., 2001 Genes Dev 15:1493-1505; Hudson et al., 1997 Cell 91:397-405) and in mouse (Kanai-Azuma et al., 2002 Development 129:2367-2379) lay a foundation for how one might attempt to approach the development of a specific germ layer cell type in the culture dish using human embryonic stem cells. There are two aspects associated with in vitro ESC culture that pose major obstacles in the attempt to recapitulate development in the culture dish. First, organized germ layer or organ structures are not produced. The majority of germ layer and organ specific genetic markers will be expressed in a heterogeneous fashion in the differentiating hESC culture system. Therefore it is difficult to evaluate formation of a specific tissue or cell type due to this lack of organ specific boundaries. Almost all genes expressed in one cell type within a particular germ layer or tissue type are expressed in other cells of different germ layer or tissue types as well. Without specific boundaries there is considerably less means to assign gene expression specificity with a small sample of 1-3 genes. Therefore one must examine considerably more genes, some of which should be present as well as some that should not be expressed in the particular cell type of the organ or tissue of interest. Second, the timing of gene expression patterns is crucial to movement down a specific developmental pathway.

- [060] To further complicate matters, it should be noted that stem cell differentiation *in vitro* is rather asynchronous, likely considerably more so than *in vivo*. As such, one group of cells may be expressing genes associated with gastrulation, while another group maybe starting final differentiation. Furthermore, manipulation of hESC monolayers or embryoid bodies (EBs) with or without exogenous factor application may result in profound differences with respect to overall gene expression pattern and state of differentiation. For these reasons, the application of exogenous factors must be timed according to gene expression patterns within a heterogeneous cell mixture in order to efficiently move the culture down a specific differentiation pathway.
- 10 [061] In one embodiment, the cells treated with the inhibitor of the PI3-kinase pathway are mesendoderm cells, which can be further differentiated into mesoderm or endoderm cells. As used herein, "mesendoderm cells" are defined by the expression of one or more genes such as, but not limited to, brachyury, goosecoid, twist, Lim-1, and GATA5, which are expressed by endoderm and mesoderm precursors, however the cells do not express SOX-17. In another embodiment, the cells treated with the inhibitor of the PI3-kinase pathway are further differentiated into endoderm cells, which can be partially or terminally differentiated endoderm cells. In one embodiment, the cells treated with the inhibitor of the PI3-kinase pathway are further differentiated into cells of the definitive endoderm lineage.
- 20 [062] As used herein, the term "endoderm" includes, but is not limited to, definitive endoderm; parietal endoderm, visceral endoderm, and mesendoderm cells. As used herein, the term "definitive endoderm" refers to early endoderm cells that have the capacity to differentiate into any or many of the endoderm cell types that are generated from the endoderm lineages in the embryo (i.e. pancreas, liver, lung, stomach, intestine and thyroid). Definitive endoderm cells are multipotent. Therefore, the use of the term "definitive endoderm" in the context of the present invention means that the cell is at least more differentiated towards an endoderm cell type than the pluripotent cell from which it is derived. Also, as used herein, producing an endoderm cell encompasses the production of a cell culture that is enriched for endoderm cells.
- 25 [063] As used herein, "definitive endoderm" cells are characterized by the expression of specific marker transcripts such as SOX17, with the concomitant absence of marker transcripts for AFP and thrombomodulin. In addition, such cells can express MIX1, GATA4, HNFalpha, and HNF3b. Additionally, LY 294002 treatment results in the loss of a subset of cell surface CD markers initially expressed by undifferentiated
- 30

hES cells, including, but not limited to, CD9, 27, 30, 46, 58 and 81. In some embodiments of the present invention, definitive endoderm cells express the SOX17 marker gene at a level higher than that of SOX7, a marker gene characteristic of visceral endoderm. Additionally, in certain embodiments, expression of the SOX17 marker gene is higher than the expression of the OCT4 marker gene, which is characteristic of hESCs. In other embodiments of the present invention, definitive endoderm cells express the SOX17 marker gene at a level higher than that of the AFP, SPARC or Thrombomodulin (TM) marker genes. In certain embodiments of the present invention, the definitive endoderm cells produced by the methods described herein do not express PDX1 (PDX1-negative). In another embodiment, the cells display similarly low expression of thrombomodulin as seen in a population of pluripotent cells as determined, for example, by flow cytometry.

[064] In certain embodiments of the present invention, the definitive endoderm cell cultures produced by the methods described herein are substantially free of cells expressing the OCT4, SOX7, AFP, SPARC, TM, ZIC1 or BRACH marker genes. In other embodiments, the definitive endoderm cell cultures produced by the methods described herein are substantially free of cells expressing the SOX7, AFP, SPARC, TM, ZIC1 or BRACH marker genes. With respect to cells in cell cultures, the term “substantially free of” means that the specified cell type is present in an amount of less than about 5% of the total number of cells present in the cell culture.

[065] As used herein, the term “differentiate” refers to the production of a cell type that is more differentiated than the cell type from which it is derived. The term therefore encompasses cell types that are partially and terminally differentiated.

[066] In certain embodiments of the present invention, the term “enriched” refers to a cell culture that contains more than approximately 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% of the desired cell lineage.

[067] The cell types that differentiate from embryonic stem cells after contact with inhibitors of the PI3-kinase pathway and the member of the TGF $\beta$  family have several uses in various fields of research and development including but not limited to drug discovery, drug development and testing, toxicology, production of cells for therapeutic purposes as well as basic science research. These cell types express molecules that are of interest in a wide range of research fields. These include the molecules known to be required for the functioning of the various cell types as described in standard reference texts. These molecules include, but are not limited to,

cytokines, growth factors, cytokine receptors, extracellular matrix, transcription factors, secreted polypeptides and other molecules, and growth factor receptors.

[068] In one embodiment, the pluripotent cell is a human cell. As used herein, the term "pluripotent human cell" encompasses pluripotent cells obtained from human embryos, fetuses or adult tissues. In one preferred embodiment, the pluripotent human cell is a human pluripotent embryonic stem cell. In another embodiment the pluripotent human cell is a human pluripotent fetal stem cell, such as a primordial germ cell. In another embodiment the pluripotent human cell is a human pluripotent adult stem cell. As used herein, the term "pluripotent" refers to a cell capable of at least developing into one of ectodermal, endodermal and mesodermal cells. As used herein the term "pluripotent" refers to cells that are totipotent and multipotent. As used herein, the term "totipotent cell" refers to a cell capable of developing into all lineages of cells. The term "multipotent" refers to a cell that is not terminally differentiated. As also used herein, the term "multipotent" refers to a cell that, without manipulation (i.e., nuclear transfer or dedifferentiation inducement), is incapable of forming differentiated cell types derived from all three germ layers (mesoderm, ectoderm and endoderm), or in other words, is a cell that is partially differentiated. The pluripotent human cell can be selected from the group consisting of a human embryonic stem (ES) cell; a human inner cell mass (ICM)/epiblast cell; a human primitive ectoderm cell, such as an early primitive ectoderm cell (EPL); a human primordial germ (EG) cell; and a human teratocarcinoma (EC) cell. The human pluripotent cells of the present invention can be derived using any method known to those of skill in the art. For example, the human pluripotent cells can be produced using de-differentiation and nuclear transfer methods. Additionally, the human ICM/epiblast cell or the primitive ectoderm cell used in the present invention can be derived *in vivo* or *in vitro*. EPL cells may be generated in adherent culture or as cell aggregates in suspension culture, as described in WO 99/53021. Furthermore, the human pluripotent cells can be passaged using any method known to those of skill in the art, including, manual passaging methods, and bulk passaging methods such as antibody selection and protease passaging.

[069] In certain embodiment, the embryonic stem cell of the invention has a normal karyotype, while in other embodiments, the embryonic stem cell has an abnormal karyotype. In one embodiment, a majority of the embryonic stem cells have an abnormal karyotype. It is contemplated that greater than 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90% or greater than 95% of metaphases examined will display

an abnormal karyotype. In certain embodiments, the abnormal karyotype is evident after the cells have been cultured for greater than 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 20 passages. In one embodiment, the abnormal karyotype comprises a trisomy of at least one autosomal chromosome, wherein the autosomal chromosome is selected from the group consisting of chromosomes 1, 7, 8, 12, 14, and 17. In another embodiment, the abnormal karyotype comprises a trisomy of more than one autosomal chromosome, wherein at least one of the more than one autosomal chromosomes is selected from the group consisting of chromosomes 1, 7, 8, 12, 14, and 17. In one embodiment, the autosomal chromosome is chromosome 12 or 17. In another embodiment, the abnormal karyotype comprises an additional sex chromosome. In one embodiment, the karyotype comprises two X chromosomes and one Y chromosome. It is also contemplated that translocations of chromosomes may occur, and such translocations are encompassed within the term "abnormal karyotype." Combinations of the foregoing chromosomal abnormalities are also encompassed by the invention.

[070] As recited above, the invention encompasses a method of differentiating a pluripotent mammalian cell comprising: (a) providing the pluripotent mammalian cell, and (b) contacting the pluripotent mammalian cell with an effective amount of an inhibitor of the PI3-kinase signaling pathway and the member of the TGF $\beta$  family to at least partially differentiate the pluripotent cell to a cell of the endoderm lineage. In one embodiment, step (b) comprises the use of a cell differentiation environment. In another embodiment, the cells can be contacted with a cell differentiation environment after step (b).

[071] As used herein, the term "cell differentiation environment" refers to a cell culture condition wherein the pluripotent cells are induced to differentiate, or are induced to become a human cell culture enriched in differentiated cells. Preferably, the differentiated cell lineage induced by the growth factor will be homogeneous in nature. The term "homogeneous," refers to a population that contains more than approximately 50%, 60%, 70%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% of the desired cell lineage.

[072] In one embodiment, the pluripotent cells are induced to differentiate into cells of a lineage selected from the group consisting of mesendoderm, endoderm and mesoderm lineages.. In a further embodiment, the pluripotent cells are induced to differentiate into cells of the definitive endoderm lineage. Preferably, the pluripotent cells are induced to differentiate into a population comprising greater than

approximately 50% definitive endoderm cells. In other embodiments, the population comprises greater than approximately 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% of the definitive endoderm lineage.

5     **[073]**           A differentiating medium or environment may be utilized to partially, terminally, or reversibly differentiate the pluripotent cells of the present invention, either prior to, during, or after contacting the pluripotent cells with an inhibitor of PI3-kinase and the member of the TGF $\beta$  family. In accordance with the invention the medium of the cell differentiation environment may contain a variety of components  
10     including, for example, KODMEM medium (Knockout Dulbecco's Modified Eagle's Medium), DMEM, Ham's F12 medium, FBS (fetal bovine serum), FGF2 (fibroblast growth factor 2), KSR, serum, or hLIF (human leukemia inhibitory factor). The cell differentiation environment can also contain supplements such as L-Glutamine, NEAA (non-essential amino acids), P/S (penicillin/streptomycin), N2 and  $\beta$ -mercaptoethanol  
15     ( $\beta$ -ME). It is contemplated that additional factors may be added to the cell differentiation environment, including, but not limited to, fibronectin, laminin, heparin, heparin sulfate, retinoic acid, members of the epidermal growth factor family (EGFs), members of the fibroblast growth factor family (FGFs) including FGF2 and/or FGF8, members of the platelet derived growth factor family (PDGFs), antagonists of the  
20     transforming growth factor (TGF)/ bone morphogenetic protein (BMP)/ growth and differentiation factor (GDF) factor families including but not limited to noggin, follistatin, chordin, gremlin, cerberus/DAN family proteins, ventropin, high dose activin, and amnionless. TGF/BMP/GDF antagonists could also be added in the form of TGF/BMP/GDF receptor-Fc chimeras. Other factors that may be added include  
25     molecules that can activate or inactivate signaling through Notch receptor family, including but not limited to proteins of the Delta-like and Jagged families as well as inhibitors of Notch processing or cleavage. Other growth factors may include members of the insulin like growth factor family (IGF), insulin, the wingless related (WNT) factor family, and the hedgehog factor family. Additional factors may be added to  
30     promote mesendoderm stem/progenitor, endoderm stem/progenitor, mesoderm stem/progenitor, or definitive endoderm stem/progenitor proliferation and survival as well as survival and differentiation of derivatives of these progenitors.

**[074]**           In other embodiments, the cell differentiation environment comprises plating the cells in an adherent culture. As used herein, the terms "plated" and

“plating” refer to any process that allows a cell to be grown in adherent culture. As used herein, the term “adherent culture ” refers to a cell culture system whereby cells are cultured on a solid surface, which may in turn be coated with a solid substrate that may in turn be coated with another surface coat of a substrate, such as those listed below, or any other chemical or biological material that allows the cells to proliferate or be stabilized in culture. The cells may or may not tightly adhere to the solid surface or to the substrate. In one embodiment, the cells are plated on matrigel-coated plates. The substrate for the adherent culture may comprise any one or combination of polyornithine, laminin, poly-lysine, purified collagen, gelatin, extracellular matrix, fibronectin, tenascin, vitronectin, entactin, heparin sulfate proteoglycans, poly glycolytic acid (PGA), poly lactic acid (PLA), poly lactic-glycolic acid (PLGA) and feeder layers such as, but not limited to, primary fibroblasts or fibroblast cells lines. Furthermore, the substrate for the adherent culture may comprise the extracellular matrix laid down by a feeder layer, or laid down by the pluripotent human cell or cell culture.

[075] The methods of the present invention contemplate that cells may be cultured with a feeder cell or feeder layer. As used herein, a “feeder cell” is a cell that is co-cultured with a target cell and stabilizes the target cell in its current state of differentiation. A feeder layer comprises more than one feeder cell in culture. In one embodiment of the above method, conditioned medium is obtained from a feeder cell that stabilizes the target cell in its current state of differentiation. Any and all factors produced by a feeder cell that allow a target cell to be stabilized in its current state of differentiation can be isolated and characterized using methods routine to those of skill in the art. These factors may be used in lieu of a feeder layer, or may be used to supplement a feeder layer.

[076] As used herein, the term "stabilize" refers to the differentiation state of a cell. When a cell or cell population is stabilized, it will continue to proliferate over multiple passages in culture, and preferably indefinitely in culture; additionally, each cell in the culture is preferably of the same differentiation state, and when the cells divide, typically yield cells of the same cell type or yield cells of the same differentiation state. Preferably, a stabilized cell or cell population does not further differentiate or de-differentiate if the cell culture conditions are not altered, and the cells continue to be passaged and are not overgrown. Preferably the cell that is stabilized is capable of proliferation in the stable state indefinitely, or for at least more



than 2 passages. Preferably, it is stable for more than 5 passages, more than 10 passages, more than 15 passages, more than 20 passages, more than 25 passages, or most preferably, it is stable for more than 30 passages. In one embodiment, the cell is stable for greater than 1 year of continuous passaging.

5 [077] In one embodiment, stem cells are maintained in culture in a pluripotent state by routine passage until it is desired that they be differentiated into definitive endoderm. It is contemplated that a member of the TGF $\beta$  family is administered to the pluripotent cell in conjunction with the inhibitor of the PI3-kinase pathway. As used herein, the term "member of the TGF- $\beta$  family" refers to growth factors that are  
10 generally characterized by one of skill in the art as belonging to the TGF- $\beta$  family, either due to homology with known members of the TGF- $\beta$  family, or due to similarity in function with known members of the TGF- $\beta$  family. In certain embodiments, the member of the TGF- $\beta$  family is selected from the group consisting of Nodal, Activin A, Activin B, TGF- $\beta$ , BMP2, BMP4, and mixtures of the foregoing. In one embodiment,  
15 the member of the TGF- $\beta$  family is Activin A. Additionally, the growth factor Wnt3a is useful for the production of definitive endoderm cells. In certain embodiments of the present invention, combinations of any of the above-mentioned growth factors can be used. It is not necessary that these components be added to the cells simultaneously.

[078] With respect to some of the embodiments of differentiation methods  
20 described herein, the above-mentioned growth factors are provided to the cells so that the growth factors are present in the cultures at concentrations sufficient to promote differentiation of at least a portion of the stem cells to definitive endoderm. In some embodiments of the present invention, the above-mentioned growth factors are present in the cell culture at a concentration of at least about 10 ng/ml, at least about 25 ng/ml,  
25 at least about 50 ng/ml, at least about 75 ng/ml, at least about 100 ng/ml, at least about 200 ng/ml, at least about 300 ng/ml, at least about 400 ng/ml, at least about 500 ng/ml, or at least about 1000 ng/ml.

[079] In certain embodiments of the present invention, the above-mentioned growth factors are removed from the cell culture subsequent to their addition. For  
30 example, the growth factors can be removed within about one day, about two days, about three days, about four days, about five days, about six days, about seven days, about eight days, about nine days or about ten days after their addition. In a preferred embodiment, the growth factors are removed about four days after their addition.

[080] Cultures of definitive endoderm cells can be grown in medium containing reduced serum or no serum. In certain embodiments of the present invention, serum concentrations can range from about 0.1% to about 20% (v/v). In some embodiments, definitive endoderm cells are grown with serum replacement. In other embodiments, definitive endoderm cells are grown in the presence of B27. In such embodiments, the concentration of B27 supplement can range from about 0.2% to about 20% (v/v) or greater than about 20% (v/v). Alternatively, the concentration of the added B27 supplement can be measured in terms of multiples of the strength of a commercially available B27 stock solution. For example, B27 is available from Invitrogen (Carlsbad, CA) as a 50X stock solution. Addition of a sufficient amount of this stock solution to a sufficient volume of growth medium produces a medium supplemented with the desired amount of B27. For example, the addition of 10 ml of 50X B27 stock solution to 90 ml of growth medium would produce a growth medium supplemented with 5X B27. The concentration of B27 supplement in the medium can be about 0.1X, about 0.2X, about 0.3X, about 0.4X, about 0.5X, about 0.6X, about 0.7X, about 0.8X, about 0.9X, about 1X, about 1.1X, about 1.2X, about 1.3X, about 1.4X, about 1.5X, about 1.6X, about 1.7X, about 1.8X, about 1.9X, about 2X, about 2.5X, about 3X, about 3.5X, about 4X, about 4.5X, about 5X, about 6X, about 7X, about 8X, about 9X, about 10X, about 11X, about 12X, about 13X, about 14X, about 15X, about 16X, about 17X, about 18X, about 19X, about 20X and greater than about 20X.

[081] The progression of the hESC culture to definitive endoderm can be monitored by quantitating expression of marker genes characteristic of definitive endoderm as well as the lack of expression of marker genes characteristic of hESCs and other cell types. One method of quantitating gene expression of such marker genes is through the use of quantitative PCR (Q-PCR). Methods of performing Q-PCR are well known in the art. Other methods that are known in the art can also be used to quantitate marker gene expression. Marker gene expression can be detected by using antibodies specific for the marker gene of interest.

[082] Using the methods described herein, compositions comprising definitive endoderm cells substantially free of other cell types can be produced. Alternatively, compositions comprising mixtures of hESCs and definitive endoderm cells can be produced. For example, compositions comprising at least 5 definitive endoderm cells for every 95 hESCs can be produced. In other embodiments, compositions comprising

at least 95 definitive endoderm cells for every 5 hESCs can be produced. Additionally, compositions comprising other ratios of definitive endoderm cells to hESCs are contemplated.

[083] In some embodiments of the present invention, definitive endoderm cells  
5 can be isolated by using an affinity tag that is specific for such cells. One example of an affinity tag specific for definitive endoderm cells is an antibody that is specific to a marker polypeptide that is present on the cell surface of definitive endoderm cells but which is not substantially present on other cell types that would be found in a cell culture produced by the methods described herein.

10 [084] It is contemplated that the pluripotent cells can be dissociated to an essentially single cell culture prior to being contacted with the inhibitor of the PI3-kinase signaling pathway. As used herein, an "essentially single cell culture" is a cell culture wherein during passaging, the cells desired to be grown are dissociated from one another, such that the majority of the cells are single cells, or two cells that remain  
15 associated (doublets). Preferably, greater than 40%, 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99% or more of the cells desired to be cultured are singlets or doublets. The term encompasses the use of any method known now or later developed that is capable of producing an essentially single cell culture. Non-limiting examples of such methods include the use of a cell dispersal buffer, and the use of proteases such as  
20 trypsin, collagenase, dispase, tripLE, and accutase. These proteases and combinations of certain of the proteases are commercially available. The invention contemplates that the cell culture can be dissociated to an essentially single cell culture at any point during passaging, and it is not necessary that the dissociation occur during the passage immediately prior to contact with the inhibitor. The dissociation can occur during one  
25 or more passages.

[085] The cells produced using the methods of the present invention have a variety of uses. In particular, the cells can be used as a source of nuclear material for nuclear transfer techniques and used to produce cells, tissues or components of organs for transplant. For example, if a definitive endoderm cell is produced, it can be used in  
30 human cell therapy or human gene therapy to treat diseases such as type 1 diabetes, liver diseases, and any other diseases that affect the esophagus, stomach, small intestines, large intestines, lungs, thymus, parathyroid and thyroid glands, gall bladder and pancreas. In one embodiment of the foregoing, the definitive endoderm cell is used to treat diabetes.

[086] Throughout this application, various publications are referenced. The disclosures of all of these publications and those references cited within those publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

5

## EXAMPLES

### Example 1

#### *Culture of Human ES cells*

##### 10 Routine Human ES cell culture

[087] The human embryonic stem cell line BG01 (BresaGen, Inc., Athens, GA) was used in this work. BG01 cells were grown in hES Medium, consisting of DMEM/F-12 (50/50) supplemented with 20% knockout serum replacer (KSR; Invitrogen), 0.1 mM MEM Non-essential amino acids (NEAA; Invitrogen), 2 mM L-  
15 Glutamine (Invitrogen), 50 U/ml penicillin, 50 µg/ml streptomycin (Invitrogen), 4 ng/ml bFGF (Sigma) and 0.1mM β-mercaptoethanol (Sigma). The cells were grown on mouse primary embryonic fibroblast feeder layers that were mitotically inactivated with mitomycin C. Feeder cells were plated at  $1.2 \times 10^6$  cells per 35 mm dish. The BG01 cells were passaged using a collagenase/trypsin method. Briefly, medium was removed  
20 from the dish, 1 ml of 200 U/ml Collagenase type IV (GibcoBRL) was added, and the cells were incubated at 37°C for 1-2 minutes. Collagenase was removed and 1 ml of 0.05% trypsin/0.53mM EDTA (GIBCO) was applied. Cells were incubated at 37°C for 30 seconds and then washed from the feeder layer, and the trypsin was inactivated in DMEM/F-12 with 10% fetal bovine serum (FBS; Hyclone). Cells were replated on  
25 feeder layers at 100,000 – 300,000 cells per 35 mm dish and were passaged every 3 days.

##### Growth of BG01 cells in feeder free conditions

[088] hES medium (25mls) was conditioned overnight on mitomycin treated MEFs plated in 75cm<sup>2</sup> flasks at 56,000 cells/cm<sup>2</sup>. The MEFs were used for up to 1  
30 week with conditioned medium (CM) collection every 24 hours. CM was supplemented with an additional 8 ng/ml of hbFGF before use. Matrigel coated dishes were prepared by diluting Growth Factor Reduced BD matrigel matrix (BD Biosciences) to a final concentration of 1:30 in cold DMEM/F-12. 1ml/35mm dish was used to coat dishes for 1-2 hours at room temperature or at least overnight at 4°C.

Plates were stored up to one week at 4°C. Matrigel solution was removed immediately before use.

#### Embryoid Body Formation

- 5 [089] The BG01 cells were disaggregated using the Collagenase/trypsin method described above. Approximately 10,000 cells were suspended in 50 µl of EB medium (DMEM (Cellgro) supplemented with 10% FBS (Atlanta Biolabs), 0.1 mM NEAA, 2 mM L-Glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin), and were dropped onto a 100 mm Petri dish lid with a p200 pipette tip. Approximately 50 drops  
10 were placed per lid. The lid was placed onto the dish and 10 ml of PBS was placed in the dish. EBs were washed from the lid at 3 and 5 days, incubated with trypsin for 5 minutes at room temperature and disaggregated with a drawn out glass pipette. Cells were washed once in 1xPBS and fixed in 2% PFA/2% sucrose for 10 minutes at room temperature. Cells were then washed twice in PBS and stored in 1%BSA/PBS ready  
15 for antibody staining.

#### **Example 2**

*Treatment of HES cells with inhibitors of PI3-kinase leads to differentiation of the HES cells*

#### 20 Inhibitor Studies

- [090] BG01 cells were passaged from feeders using the collagenase/trypsin method and were plated on matrigel-coated dishes at  $1 \times 10^5$  cells/35 mm dish in conditioned medium (CM; MEF conditioned medium plus 8 ng/ml bFGF). After approximately 24 hours, the media was replaced with fresh CM, CM with inhibitor  
25 (resuspended in EtOH), CM with EtOH, or with Spontaneous Differentiation medium (hES medium minus bFGF).

- [091] In alternative methods, the BG01 cells were plated at different concentrations prior to contact with CM, CM with inhibitor and CM with EtOH. Cells were plated at the following concentrations: approximately  $5 \times 10^4$  cells/35 mm dish,  
30 approximately  $1 \times 10^5$  cells/35 mm dish, approximately  $2 \times 10^5$  cells/35 mm dish, approximately  $4 \times 10^5$  cells/35 mm dish and, approximately  $6 \times 10^5$  cells/35 mm dish.

[092] The inhibitor LY 294002 (Biomol) was used at the concentration range approximately 20-163 µM and the inhibitor Rapamycin (Calbiochem) was used at the concentration range approximately 1.5-30 nM. LY 294002 inhibits the PI3-kinase

pathway by binding to the ATP docking site of p110. Rapamycin inhibits a subset of the PI3-kinase pathway by inhibiting mTOR (mammalian target of rapamycin).

[093] Cells were grown in these conditions for approximately 72 hours with a medium change every 24 hours. Cells were harvested using the collagenase/trypsin method for flow cytometry and RT-PCR analysis and were scraped for biochemical analysis.

[094] By observing the cells using standard microscopy, it was noted that BG01 cells undergo morphological change when cultured in the presence of either LY 294002 or rapamycin. This morphological change is notably different from the change in cells undergoing spontaneous differentiation (Figures 1A-D and 2A-D). In undifferentiated cultures, individual cells were not easily discernable, being relatively small, irregular and without clearly apparent intercellular junctions at higher density. After treatment with LY 294002, however, the cells underwent marked spreading and adopted obvious epithelioid cuboidal morphologies. Individual cells were also more readily discernable at higher densities since discrete intercellular adhering junctions were evident between neighboring cells.

[095] Additionally, in this Example, in the presence of conditioned medium, cells plated at concentrations lower than approximately  $2 \times 10^5$  cells/35 mm dish displayed changes in morphology when contacted with LY 294002 or rapamycin. Cells plated at densities of approximately  $4 \times 10^5$  cells/35 mm dish or higher did not demonstrate the same morphological changes upon contact with LY 294002 or rapamycin.

### Example 3

25 *Characteristics of cells treated with inhibitors of PI3-kinase*

[096] The inhibitor studies were performed as described in Example 2.

#### Flow Cytometry

[097] For flow cytometry, the BG01 cells were washed with 1xPBS and fixed in 2% paraformaldehyde/1xPBS for 10 minutes at room temperature. The cells were then washed in 1xPBS and approximately  $2 \times 10^5$  cells were incubated with primary antibody diluted in 1%BSA/1xPBS. The primary antibodies used were anti-CD9 and anti-thrombomodulin (Cymbus Biotechnology), FITC conjugated mouse monoclonal antibodies at a 1:10 dilution. Cells were incubated at 4°C for 30 minutes and then washed twice in 1xPBS. Where appropriate, cells were resuspended in a secondary

antibody, anti-mouse Alexa-488 (Molecular Probes) diluted 1:1000 in 1%BSA/PBS, incubated at 4°C for 30 minutes, and then washed twice in 1xPBS. Cells were resuspended in 1% BSA/1xPBS and surface expression was analyzed using a Beckman Coulter FC500.

5 RNA Isolation and RT-PCR Analysis

[098] Total RNA was isolated using TRIzol Reagent (GibcoBRL). RNA was run on a 1% agarose gel containing ethidium bromide and visualized using the AlphaImagerTM 2200 Documentation and Analysis System to ensure RNA integrity. 10 µg of RNA was treated with DNase (Ambion), which was removed with DNase  
10 Inactivation Reagent (Ambion). cDNA was prepared with 500 ng of total RNA using the Superscript II Reverse transcriptase Kit (Invitrogen) using oligo(dT) primers. PCR reactions were carried out on 1 µl of cDNA. PCR products were run on a 2% agarose gel containing ethidium bromide and visualized using the AlphaImagerTM 2200 Documentation and Analysis System. PCR primer sets used were GATA4, Mix1,  
15 Msx1, AFP, HNF4alpha, eHAND, Nanog, AFP and GAPDH.

Biochemical Analysis

[099] Activity of the PI3-kinase signaling pathway was assessed by Western blot analysis. Briefly, detergent extracts were prepared from untreated and treated cell cultures, separated by SDS-PAGE and blotted to nitrocellulose. Expression of active  
20 forms of the PI3-kinase intracellular targets PKB/Akt, S6 kinase and S6 protein was then determined by probing the nitrocellulose with appropriate antibodies to phosphorylated forms of each of these proteins. Generally, 30 µg of total protein was evaluated for each sample, primary incubations were carried out at a 1:1000 dilution of antibody, and binding in each case was detected by standard ECL based methodology.

25 Q-PCR Gene Expression Assay

[0100] Real-time measurements of gene expression were analyzed for multiple marker genes at multiple time points by Q-PCR. Marker genes characteristic of the desired as well as undesired cell types were evaluated to gain a better understanding of the overall dynamics of the cellular populations. The strength of Q-PCR analysis  
30 includes its extreme sensitivity and relative ease of developing the necessary markers, as the genome sequence is readily available. Furthermore, the extremely high sensitivity of Q-PCR permits detection of gene expression from a relatively small number of cells within a much larger population. In addition, the ability to detect very low levels of gene expression may provide indications for “differentiation bias” within

the population. The bias towards a particular differentiation pathway, prior to the overt differentiation of those cellular phenotypes, would likely be unrecognizable using immunocytochemical techniques. For this reason, Q-PCR provides a method of analysis that is complementary to immunocytochemical techniques for screening the success of differentiation treatments. This tool provides a means of evaluating our differentiation protocol successes in a quantitative format at semi-high throughput scales of analysis.

[0101] The general approach was to perform relative quantitation using SYBR Green chemistry on the Rotor Gene 3000 instrument (Corbett Research) and a two-step RT-PCR format. Primers were designed to lie over exon-exon boundaries or span introns of at least 800 bp when possible, as this has been empirically determined to eliminate amplification from contaminating genomic DNA. When marker genes were employed that do not contain introns or they possess pseudogenes, DNase I treatment of RNA samples was performed. The markers relevant for the early phases of hESC differentiation (specifically ectoderm, mesoderm, definitive endoderm and extra-embryonic endoderm) and for which validated primer sets exist are provided below in Table 1. The human specificity of these primer sets has also been demonstrated. This is an important fact since the hESCs were often grown on mouse feeder layers. Most typically, triplicate samples were taken for each condition and independently analyzed in duplicate to assess the biological variability associated with each quantitative determination.

[0102] Total RNA was isolated using RNeasy (Qiagen) and quantitated using RiboGreen (Molecular Probes). Reverse transcription from 350-500 ng of total RNA was carried out using the iScript reverse transcriptase kit (BioRad), which contains a mix of oligo-dT and random primers. Each 20  $\mu$ L reaction was subsequently diluted up to 100  $\mu$ L total volume and 3  $\mu$ L was used in each 10  $\mu$ L Q-PCR reaction containing 400 nM forward and reverse primers and 5  $\mu$ L 2X SYBR Green master mix (Qiagen). Two step cycling parameters were used employing a 5 second denature at 85-94°C (specifically selected according to the melting temp of the amplicon for each primer set) followed by a 45 second anneal/extend at 60°C. Fluorescence data was collected during the last 15 seconds of each extension phase. A three point, 10-fold dilution series was used to generate the standard curve for each run and cycle thresholds (Ct's) were converted to quantitative values based on this standard curve. The quantitated



values for each sample were normalized to housekeeping gene performance and then average and standard deviations were calculated for triplicate samples. At the conclusion of PCR cycling, a melt curve analysis was performed to ascertain the specificity of the reaction. A single specific product was indicated by a single peak at the  $T_m$  appropriate for that PCR amplicon. In addition, reactions performed without reverse transcriptase served as the negative control and do not amplify.

[0103] Both Cyclophilin G and GUS were used to calculate a normalization factor for all samples. The use of multiple HGs simultaneously reduces the variability inherent to the normalization process and increases the reliability of the relative gene expression values (Vandesompele, et al., 2002, Genome Biol., 3:RESEARCH0034).

[0104] Q-PCR was used to determine the relative gene expression levels of many marker genes across samples receiving different experimental treatments. The marker genes employed have been chosen because they exhibit enrichment in specific populations representative of the early germ layers and in particular have focused on sets of genes that are differentially expressed in definitive endoderm and extra-embryonic endoderm. These genes as well as their relative enrichment profiles are highlighted in Table 1.

TABLE 1

Germ Layer	Gene	Expression Domains
Endoderm	SOX17 MIXL1 GATA4 HNF3b  GSC Cerebrus	definitive, visceral and parietal endoderm endoderm and mesoderm definitive and primitive endoderm definitive endoderm and primitive endoderm, mesoderm, neural plate mesendoderm and definitive endoderm primitive and definitive endoderm
Extra-embryonic	SOX7 AFP SPARC TM NODAL	visceral endoderm visceral endoderm, liver parietal endoderm parietal endoderm/trophectoderm Epiblast and anterior visceralendoderm
Ectoderm	ZIC1 SOX1	neural tube, neural progenitors neural progenitors
Mesoderm	BRACH	nascent mesoderm

ImmunohistochemistrySOX17 Antibody

5 [0105] SOX17 is expressed throughout the definitive endoderm as it forms during gastrulation and its expression is maintained in the gut tube (although levels of expression vary along the A-P axis) until around the onset of organogenesis. SOX17 is also expressed in a subset of extra-embryonic endoderm cells. No expression of this protein has been observed in mesoderm or ectoderm. As such, SOX17 is an appropriate marker for the definitive endoderm lineage when used in conjunction with markers to  
10 exclude extra-embryonic lineages.

[0106] A SOX17 antibody was generated as described in U.S. Patent Application Publication No. 2005/0158853, entitled "Definitive Endoderm," hereby incorporated by reference in its entirety. Briefly, a portion of the human SOX17 cDNA corresponding to amino acids 172-414 in the carboxyterminal end of the SOX17 protein  
15 was used for production of SOX17 antibody by genetic immunization in rats at the antibody production company, GENOVAC (Freiberg, Germany), according to procedures developed there. Procedures for genetic immunization can be found in US Patent Nos. 5,830,876, 5,817,637, 6,165,993, 6,261,281 and International Publication No. WO99/13915, the disclosures of which are incorporated herein by reference in their  
20 entirety. The antibody was determined to be specific for SOX17 by both Western blot and ICC on hSOX17 cDNA transfected cell lines.

[0107] Cells to be immunostained were grown on chamber slides, and were rinsed once with 1XPBS and fixed for 10 minutes in 4% PFA/4% sucrose in PBS pH 7.4 at room temperature. They were then rinsed 3X in 1XPBS and blocked in 3% goat  
25 serum with 0.1% Triton-X100 in PBS for 1 hour at room temperature. Primary antibodies were diluted in 3% goat serum in PBS and this solution was applied overnight at 4°C. The primary antibodies used were rabbit anti-human AFP (Zymed), used at a 1:50 dilution, and rat anti- human SOX17 (obtained from Cythera, Inc.), used at 1:1000 dilution. Cells were washed for 1 hour with 3 changes of 1XPBS. Secondary  
30 antibodies were applied for 2 hours at room temperature. Secondary antibodies used were goat anti-rabbit Alexa Fluor 488 and goat anti-rat Alexa Fluor 594 (Molecular Probes), both at a 1:1000 dilution in 3% goat serum in 1XPBS. Cells were washed for 1 hour with 3 changes of 1XPBS. The chambers were removed and slides were mounted in VectaShield mounting medium with DAPI (Vector).

## Results

[0108] Using flow cytometry, it was noted that expression of CD9 decreased more rapidly in LY 294002 or rapamycin treated BG01 cells than in spontaneous differentiation in adherent culture, or in embryoid bodies (Figures 3A-E). In addition, expression of CD9 has been previously observed by others to influence cell proliferation, motility and adhesion.

[0109] By RT-PCR analysis, it was noted that a number of markers indicative of early differentiation were detected in cells treated with LY 294002 and rapamycin (Figures 4A and B). Notably, the markers detected when PI3-kinase signaling was blocked differed from those detected in spontaneously differentiating adherent cultures of BG01 cells (Figures 4A and 4B). For example, blocking PI3-kinase resulted in an increase in levels of HNF4alpha, GATA4, Mix1, and Msx1, and a decrease in levels of AFP in comparison to spontaneously differentiating cultures.

[0110] Additionally, the differences in cell morphology that were noted with varying densities of pluripotent cells were supported by PCR data. The effect of treatment with LY 294002 or Rapamycin may in some circumstances be dependent on cell density.

[0111] Figures 5A-G demonstrate that treatment of hES cell line BGO1 for 1-3 days with either LY 294002 or Rapamycin quantitatively induces the expression of genes strongly associated with mesendoderm (i.e., goosecoid/Brachyury/Cerebrus), the precursor to the definitive endoderm (SOX17/MIX1/goosecoid/Cerebrus) lineage. In contrast, as depicted in Figures 5H-5I, LY 294002 or Rapamycin do not induce expression of the pan-neurectodermal marker SOX1, or the pan-extraembryonic marker, SOX7.

[0112] In addition, SOX17 and AFP immunofluorescence staining of the BG01 cell line treated with LY 294002 (80  $\mu$ M) or rapamycin (30 nM) was performed (data not shown). The double-stainings demonstrated that there is no significant expression of AFP in the treated cells. Certain fields of the cell culture did express AFP. As determined by counting 500 cells, 296 cells, or 60% of the cell population that was treated with LY 294002, expressed SOX17.

[0113] Figure 6 shows flow cytometry analysis of thrombomodulin expression in human BG01 cells. Cells treated with 80  $\mu$ M of LY 294002 demonstrate decreased levels of thrombomodulin.

[0114] In addition, BG02 cells (BresaGen, Inc.) and H1 cells (WiCell) were also treated with 60  $\mu$ M LY 294002 as described in Example 2. Expression of AFP, Bry, FoxA2, GSC, Mox1, MixL1, Sox1, Sox 17, THBD, and ZIC1 were examined by Q-PCR after approximately 72 hours in culture with LY 294002. As shown in Figures 7A-T, treatment of either line with LY 294002 results in increased levels of Sox17, MixL1, Bry, FoxA2, and GSC, while AFP, Mox1, Sox1, THBD, and ZIC1 were either not substantially increased, or showed reduced expression. These results demonstrate the repeatability of the methods of the invention using other hES cell lines. Assays were performed in triplicate and are shown as +/- SEM.

10 [0115] By biochemical analysis, it was noted that the activity of Akt, S6 kinase and S6 was inhibited in cells maintained in the presence of LY 294002. Similarly, the activity of S6kinase and S6 was abolished in cells maintained in the presence of rapamycin.

[0116] Collectively these observations indicate that PI3-kinase signaling to mTOR is down-regulated in BG01 cells in the presence of these inhibitory drugs. The activity of S6 (a distal target in this signaling pathway) was diminished in BG01 cells undergoing spontaneous differentiation in adherent culture, but was abolished in inhibitor treated cells.

#### 20 **Example 4**

##### *TGF $\beta$ members substitute for conditioned medium*

[0117] BG01 cells were passaged from feeders using the collagenase/trypsin method and were plated on matrigel coated dishes at  $1 \times 10^5$  cells/35 mm dish in unconditioned hES medium (UCM). After approximately 24 hours, the media was replaced with fresh hES medium or MEF-CM, along with LY 294002, Activin A, or Nodal as shown in Figure 8A. LY 294002 was used at a concentration of approximately 60  $\mu$ M; Activin A was used at a concentration of approximately 100 ng/ml; and Nodal was used at a concentration of approximately 1  $\mu$ g/ml. Cells were treated for approximately 4 days, with a change in medium every 24 hours. Cells were harvested using the collagenase/trypsin method for Q-PCR analysis. Sox-17 Q-PCR was performed as described in Example 3.

[0118] Figure 8A provides Q-PCR results, showing Sox17 levels as the fold-increase over untreated cells. Significant Sox17 expression was observed in hES cells

treated with (1) CM and LY 294001, (2) UCM, LY 294002, and Activin A, and (3) UCM, LY 294002 and Nodal, but not in the absence of CM, Activin or Nodal (Figure 8A). The results indicate that MEF-CM or Activin/Nodal are necessary for LY 294002-dependent hESC-DE formation. Assays were performed in triplicate and are shown as  
5 +/- SEM.

[0119] Sox 17 expression was evaluated by Q-PCR under culture conditions competent to support hESC-DE formation (MEF-CM and LY 294002) in the presence or absence of Chordin (approximately 500 ng/ml), Follistatin (approximately 500 ng/ml), Lefty-A (approximately 500 ng/ml), Noggin (approximately 500 ng/ml), SB-  
10 43152 (approximately 10  $\mu$ M), and SU-5402 (approximately 5  $\mu$ M). Assays were performed in triplicate and are shown as +/- SEM. Figure 8B shows Sox17 levels as the fold-increase over untreated cells in the various treatments, indicating that LY 294002, MEF-CM dependent Sox17 expression was suppressed by inhibitors of Activin signaling (Follistatin and SB-43152) but not by inhibitors of BMP (Chordin and  
15 Noggin), Nodal (Lefty-A) or FGF (SU-5402) signaling.

### Example 5

#### *Akt1-11 induces formation of definitive endoderm*

[0120] BG01 cells were passaged from feeders using the collagenase/trypsin  
20 method and were plated on matrigel coated dishes at  $1 \times 10^5$  cells/35 mm dish in conditioned medium (CM; MEF conditioned medium plus 8 ng/ml bFGF). After approximately 24 hours, the media was replaced with fresh CM, CM with inhibitor (resuspended in EtOH), or CM with EtOH.

[0121] The inhibitor AktI-II (Calbiochem) was dissolved in ethanol and was  
25 used at the concentration range approximately 10-40  $\mu$ M. AktI-II inhibits the PI3-kinase pathway by directly inhibiting Akt I.

[0122] Cells were grown in these conditions for approximately 72 hours with a medium change every 24 hours. Cells were harvested using the collagenase/trypsin method for Q-PCR as described above. Figure 9 shows Sox17 expression as a fold-  
30 increase relative to untreated hES cells.

[0123] By Q-PCR, it was ascertained that the addition of approximately 40  $\mu$ M of the Akt inhibitor AKT1-II reproduces the effects of LY 294002 in promoting DE formation (Figure 9).

**Example 6**

*GSK3 activation is required for LY 294002 induced definitive endoderm formation*

[0124] Since Akt phosphorylates and inhibits GSK3 activation, and since  
5 blocking Akt promotes DE formation, we investigated whether GSK3 activation is necessary for DE formation.

[0125] BG01 cells were passaged on matrigel using the collagenase/trypsin method as described above and were plated on matrigel-coated chamber slides in CM. After approximately 24 hours, the medium was changed, and the cells were treated with  
10 60  $\mu$ M LY 294002, BIO, meBIO, or DMSO for approximately 3-4 days. The inhibitor BIO (Dr. Ali Brivanlou) was diluted in DMSO and was used at a concentration of approximately 0.1-5  $\mu$ M. MeBIO (Dr. Ali Brivanlou) was also diluted in DMSO and used at a concentration of approximately 1-5  $\mu$ M. Assays were performed in triplicate and are shown as +/- SEM.

15 [0126] Cells were grown in these conditions for approximately 3-4 days with a medium change every 24 hours. Cells were harvested using the collagenase/trypsin method for Q-PCR as described above

[0127] Addition of the GSK3 inhibitor, BIO, but not meBio, blocked LY 294002 induced DE formation as determined by Sox17 expression (Figure 10).

20 [0128] In addition, knockdown of GSK3 expression with two specific RNAi molecules, but not a mutant RNAi, blocks the ability of LY 294002 to promote DE formation. BG01 cells were passaged from feeders using the collagenase/trypsin method and were plated on matrigel coated chamber slides in ...CM. After approximately 24 hours, the medium was changed, and the cells were transfected with  
25 various RNAi sequences. The cells were transfected with 100 nM of the RNAi duplexes with Lipofectamine 2000 (Invitrogen). GSK3 $\beta$  wild-type 1 and 2 were purchased from Invitrogen (GSK3beta Validated RNAi DuoPack; # 45-1488) as was the control mutant RNAi (Invitrogen, #46-2001).

[0129] 12 hours after transfection, LY 294002 was added to all wells. The  
30 medium was changed daily. Cells were grown in these conditions for approximately 1-4 days, and the cells were subsequently fixed and immunostained at days 1, 2, 3, and 4 as indicated in Figures 11A and B.

[0130] Cells to be immunostained were rinsed once with 1XPBS and fixed for 10 minutes in 4% PFA/4% sucrose in PBS pH 7.4 at room temperature. They were then rinsed 3X in 1XPBS and blocked in 3% goat serum with 0.1% Triton-X100 in PBS for 1 hour at room temperature. Primary antibodies were diluted in 3% goat serum in PBS and this solution was applied overnight at 4°C. The primary antibodies used were pan GSK (BD Biosciences, Cat. # 610202), used at a 1:1000 dilution and rat anti-human SOX17 (obtained from Cythera, Inc.), used at 1:1000 dilution. Cells were washed for 1 hour with 3 changes of 1XPBS. Secondary antibodies were applied for 2 hours at room temperature. Secondary antibodies used were goat anti-rabbit Alexa Fluor 488 and goat anti-rat Alexa Fluor 594 (Molecular Probes), both at a 1:1000 dilution in 3% goat serum in 1XPBS. Cells were washed for 1 hour with 3 changes of 1XPBS. The chambers were removed and slides were mounted in VectaShield mounting medium with DAPI (Vector). Greater than 200 cells per sample were scored, and assays were performed in duplicate.

[0131] Figures 11A and B show the percentage of GSK3 and Sox17 positive cells, respectively, upon treatment with the different RNAi molecules. Treatment with the GSK3 specific RNAi sequences decreased expression of both GSK3 and Sox17, while treatment with the control RNAi sequence did not decrease expression of either GSK3 or Sox17. Therefore, GSK3 activation is necessary for the induction of DE by LY 294002.

[0132] These results indicate that the endogenous activity of PI3-kinase and a member of the TGF $\beta$  family may be necessary for the pluripotency of human ES cells, and that pharmacological inhibition of PI3-kinase activity in self-renewing populations results in a biological commitment to multilineage differentiation *in vitro*. These data indicate that commitment to differentiation to definitive endoderm in human ES cells may be controlled by the level of endogenous PI3-kinase, and that this pivotal signaling axis must be silenced for the onset of differentiation to definitive endoderm to occur *in vitro*. Suppression of this kinase activity has been achieved with highly specific, potent and well documented pharmacological inhibitors, LY 294002 in the case of PI3-kinase (Pullen & Thomas, (1997) FEBS Lett., 410: 78-82), rapamycin in the case of mTOR (Han et al., (1995) J. Biol. Chem., 21396-21403), and AktI-II in the case of Akt I. The optimal effective concentrations for initiating human ES cell differentiation *in vitro* by each of these inhibitors has been established. Ongoing studies are aimed at defining the subset of serum factors other than TGF $\beta$  family members that are essential for

maintaining the endogenous activation of both PI3-kinase and p70 S6 kinase in the context of human ES *in vitro* pluripotency.

### Example 7

#### 5 *Kidney capsule assays of LY 294002 induced definitive endoderm cells*

[0133] Untreated hESCs or hESCs treated for 4 days with 60  $\mu$ M LY 294002 were collagenase treated to generate cell aggregates (~ 50 cells /aggregate), washed in warm media, then gently resuspended in 2 ml DMEM/F12, 10% FCS and left overnight at 37°C, 10% CO<sub>2</sub> to facilitate further aggregation. Approximately  $2.5 \times 10^6$  cells were  
 10 injected into the kidney capsule of 5 week old male SCID-beige mice. 6 weeks after transplantation, mice were sacrificed, and the kidneys were removed and fixed in 4% paraformaldehyde. Following fixation, kidneys were embedded in paraffin wax, sectioned and mounted onto glass slides in preparation for H&E and immunostaining. After mounting, slides underwent deparaffinization, rehydration and heat induced  
 15 epitope retrieval with Trilogy (Cell Marque, CMX-833). Slides were stained using the alkaline phosphatase Vectastain ABC System (Vector Labs, AK-5002) and Vector Red substrate (Vector Labs, SK-5100). The following antibodies were used for immunocytochemistry, albumin (Sigma, A0433), AFP (Cell Marque, CMC700), Gastrin (CMC106), HSF (Cell Marque, CMC773), LFABP (Fitzgerald Industries  
 20 International, RDI-FABP-L2E3), TTF-1 (Cell Marque, CMC572), Villin (Cell Marque, CMC833). For fluorescence immunocytochemistry the following secondary antibodies were used; Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes, A11001), Alexa Fluor 594 goat anti-rabbit IgG (Molecular Probes, A11012).

[0134] Figure 12A shows that LY 294002 treated HESCs formed a large mass  
 25 on the kidney (right panel), which is striking in comparison to a kidney that did not contain an implant (left panel).

[0135] , Figures 12B-G show photomicrographs of immunostainings of LY 294002 treated aggregates after culture under a kidney capsule for approximately 6 weeks. The aggregates express TTF-1 (B), AFP (C), villin (D), gastrin (E), HSA (F)  
 30 and LFABP (G), indicating that the LY 294002 treated cells differentiate to derivatives of endoderm. The expression of LFABP and albumin were largely co-localized (data not shown). Additionally, Figures 12H and 12I show Q-PCR results, showing a 1000-fold increase in FABP1 mRNA and a 4500-fold increase in albumin mRNA in



LY294002 treated HESCs relative to LY 294002 treated HESCs that were not implanted. Assays were performed in triplicate and are shown as +/- SEM.

## CLAIMS

WE CLAIM:

- 5 1. A method of differentiating a pluripotent mammalian cell comprising:
  - (a) providing the pluripotent mammalian cell, and
  - (b) contacting the pluripotent mammalian cell with an effective amount of an inhibitor of the PI3-kinase signaling pathway and a member of the TGF $\beta$  family to at least partially differentiate the pluripotent cell to a cell of the endoderm lineage.
- 10 2. The method of Claim 1, wherein the mammalian cell is contacted with the inhibitor and the member of the TGF $\beta$  family for greater than approximately 24 hours.
- 15 3. The method of Claim 1, wherein GSK3 is activated.
4. The method of Claim 1, wherein the inhibitor is selected from the group consisting of LY 294002, Rapamycin, wortmannin, lithium chloride, Akt inhibitor I, Akt inhibitor II, Akt inhibitor III, NL-71-101, and mixtures of the foregoing.
- 20 5. The method of Claim 1, wherein the inhibitor is LY 294002.
6. The method of Claim 5, wherein LY 294002 is initially present at a concentration of approximately 1  $\mu$ M to approximately 500  $\mu$ M.
- 25 7. The method of Claim 5, wherein LY 294002 is initially present at a concentration of approximately 20  $\mu$ M to approximately 163  $\mu$ M.
8. The method of Claim 1, wherein the inhibitor is Rapamycin.
- 30 9. The method of Claim 8, wherein Rapamycin is initially present at a concentration of approximately 0.1 nM to approximately 500 nM.
10. The method of Claim 8, wherein Rapamycin is initially present at a concentration of approximately 1.5 nM to approximately 30 nM.
- 35 11. The method of Claim 1, wherein the inhibitor is AktI-II.
12. The method of Claim 11, wherein AktI-II is initially present at a concentration of approximately 10  $\mu$ M to approximately 50  $\mu$ M.
- 40

13. The method of Claim 1, wherein step (b) occurs in the presence of bFGF.
14. The method of Claim 13, wherein bFGF is initially present at a concentration of  
5 approximately 1 ng/ml to approximately 12 ng/ml.
15. The method of Claim 1, wherein the member of the TGF $\beta$  family is selected from  
the group consisting of Nodal, Activin A, Activin B, TGF- $\beta$ , BMP2, BMP4, and  
mixtures of the foregoing.
- 10 16. The method of Claim 1, wherein the member of the TGF $\beta$  family is Activin A.
17. The method of Claim 15, wherein the member of the TGF $\beta$  family is present in a  
conditioned medium.
- 15 18. The method of Claim 17, wherein the conditioned medium is obtained from a  
feeder layer of embryonic fibroblasts.
19. The method of Claim 1, wherein the mammalian pluripotent cell is selected from  
20 the group consisting of an embryonic stem cell, an ICM/epiblast cell, a primitive  
ectoderm cell, a primordial germ cell, and a teratocarcinoma cell.
20. The method of Claim 19, wherein the mammalian pluripotent cell is a human  
embryonic stem cell.
- 25 21. The method of Claim 1, wherein the cell is dissociated to an essentially single cell  
culture prior to contacting the cell with the inhibitor.
22. The method of Claim 21, wherein the cell is dissociated using a protease.
- 30 23. The method of Claim 22, wherein the protease is trypsin.
24. The method of Claim 1, wherein the pluripotent cell is plated prior to contact with  
the PI3-kinase inhibitor and the member of the TGF $\beta$  family.
- 35 25. The method of Claim 1, wherein the differentiated cell is a definitive endoderm cell.
26. A composition comprising a population of isolated differentiated mammalian cells,  
wherein the cells are differentiated from a pluripotent cell *in vitro*, and  
40 wherein greater than approximately 50% of the cells express SOX17 but do not  
express AFP.

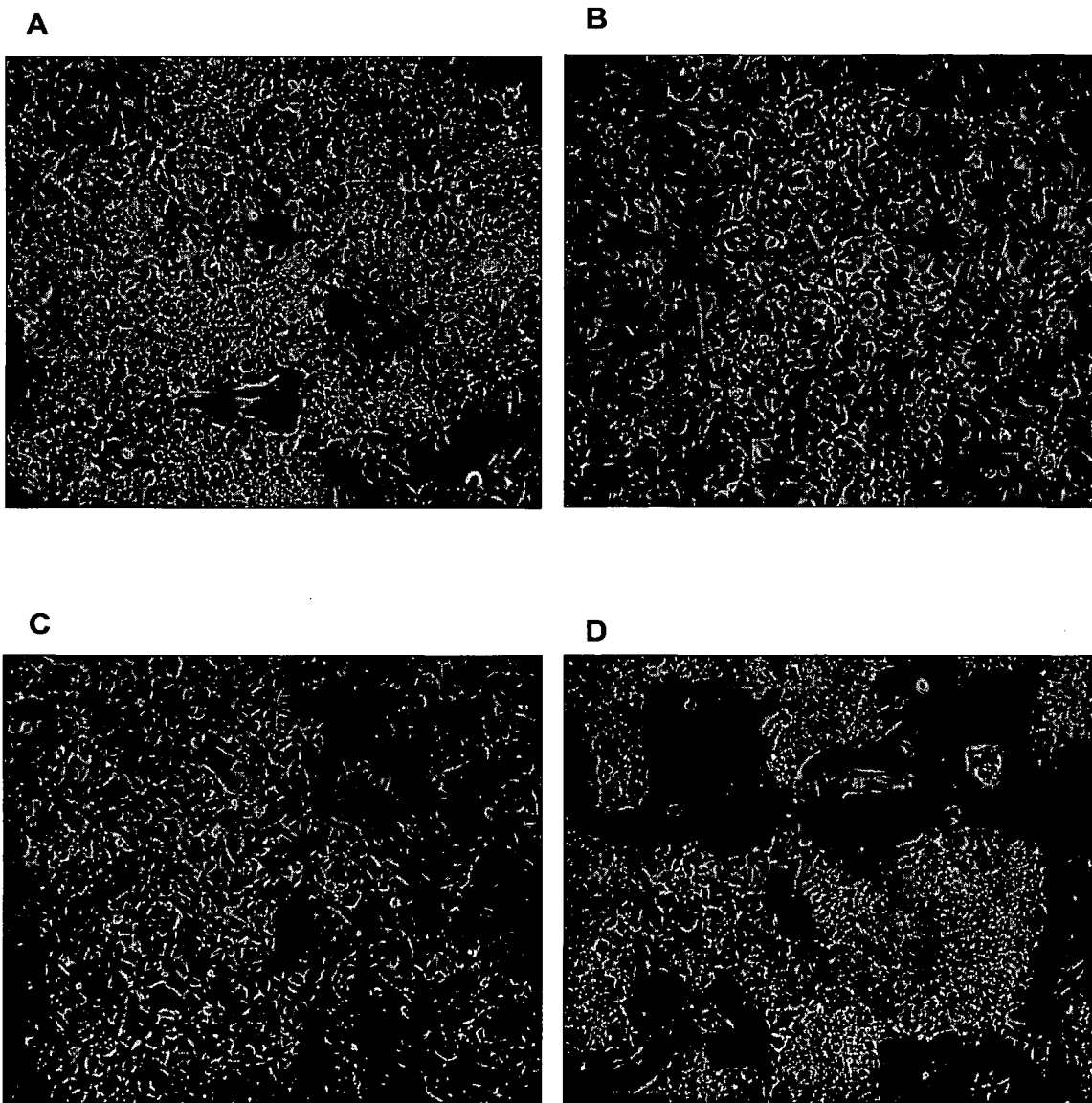
27. The composition of Claim 26, wherein greater than approximately 60% of the cells express SOX17 but do not express AFP.
28. The composition of Claim 26, wherein greater than approximately 70% of the cells  
5 express SOX17 but do not express AFP.
29. The composition of Claim 26, wherein the population has increased expression of HNF4alpha, GATA4, Mix1, and Msx1, and decreased expression of AFP in comparison to a population of spontaneously differentiating pluripotent cells.  
10
30. The composition of Claim 26, wherein the population has increased expression of Goosecoid, Brachyury, and Cerebrus, and decreased expression of AFP in comparison to a population of spontaneously differentiating pluripotent cells.
- 15 31. The composition of Claim 26, wherein the population has increased expression of MIX1, Goosecoid, and Cerebrus, and decreased expression of AFP in comparison to a population of spontaneously differentiating pluripotent cells.
- 20 32. The composition of Claim 31, wherein the population does not have increased expression of SOX1 in comparison to a population of spontaneously differentiating pluripotent cells.
- 25 33. The composition of Claim 31, wherein the population does not have increased expression of SOX7 in comparison to a population of spontaneously differentiating pluripotent cells.
34. The composition of Claim 26, wherein the cells have been differentiated by contact with a PI3-kinase inhibitor and a member of the TGFβ family.
- 30 35. The composition of Claim 34, wherein the member of the TGFβ family is selected from the group consisting of Nodal, Activin A, Activin B, TGF-β, BMP2, BMP4, and mixtures of the foregoing.
- 35 36. The composition of Claim 34, wherein the member of the TGFβ family is Activin A.
37. The composition of Claim 34, wherein the member of the TGFβ family is present in a conditioned medium.

38. The composition of Claim 34, wherein the cells are dissociated to an essentially single cell culture prior to being contacted with the inhibitor and the member of the TGF $\beta$  family.
- 5 39. The composition of Claim 38, wherein the cells are dissociated using a protease.
40. The composition of Claim 39, wherein the protease is trypsin.
41. The composition of Claim 34, wherein the cells were contacted with the PI3-kinase  
10 inhibitor and the member of the TGF $\beta$  family for greater than approximately 24 hours.
42. The composition of Claim 34, wherein the inhibitor is selected from the group consisting of LY 294002, Rapamycin, wortmannin, lithium chloride, Akt inhibitor I,  
15 Akt inhibitor II, Akt inhibitor III, NL-71-101, and mixtures of the foregoing.
43. The composition of Claim 34, wherein the inhibitor is Rapamycin.
44. The composition of Claim 34, wherein the inhibitor is LY 294002.
- 20 45. The composition of Claim 34, wherein the inhibitor is AktI-II.
46. The composition of Claim 34, wherein GSK3 is activated in at least one cell.
- 25 47. A composition comprising a homogenous population of isolated definitive endoderm cells, wherein the cells were differentiated in an *in vitro* culture, and wherein greater than approximately 50% of the population are definitive endoderm cells.
- 30 48. The composition of Claim 47, wherein greater than approximately 60% of the population are definitive endoderm cells.
49. The composition of Claim 48, wherein greater than approximately 70% of the population are definitive endoderm cells.
- 35 50. The composition of Claim 47, wherein the definitive endoderm cells express SOX17 but do not express AFP.
- 40 51. The composition of Claim 47, wherein the population has increased expression of HNF4alpha, GATA4, Mix1, and Msx1, and decreased expression of AFP in comparison to a population of spontaneously differentiating pluripotent cells.

52. The composition of Claim 47, wherein the population has increased expression of goosecoid, Brachyury, and Cerebrus, and decreased expression of AFP in comparison to a population of spontaneously differentiating pluripotent cells.
- 5 53. The composition of Claim 47, wherein the population has increased expression of MIX1, goosecoid, and Cerebrus, and decreased expression of AFP in comparison to a population of spontaneously differentiating pluripotent cells.
54. The composition of Claim 53, wherein the population does not have increased  
10 expression of SOX1 in comparison to a population of spontaneously differentiating pluripotent cells.
55. The composition of Claim 53, wherein the population does not have increased  
15 expression of SOX7 in comparison to a population of spontaneously differentiating pluripotent cells.
56. A composition, comprising a cell culture medium, an inhibitor of the PI3-kinase pathway, and the member of the TGF $\beta$  family.
- 20 57. The composition of Claim 56, wherein the inhibitor is selected from the group consisting of LY 294002, Rapamycin, wortmannin, lithium chloride, Akt inhibitor I, Akt inhibitor II, Akt inhibitor III, NL-71-101, and mixtures of the foregoing.
58. The composition of Claim 56, wherein the inhibitor is Rapamycin.
- 25 59. The composition of Claim 58, wherein Rapamycin is initially present at a concentration of approximately 0.1 nM to approximately 500 nM.
60. The composition of Claim 59, wherein Rapamycin is initially present at a  
30 concentration of approximately 1.5 nM to approximately 30 nM.
61. The composition of Claim 56, wherein the inhibitor is LY 294002.
62. The composition of Claim 61, wherein LY 294002 is initially present at a  
35 concentration of approximately 1  $\mu$ M to approximately 500  $\mu$ M.
63. The composition of Claim 62, wherein LY 294002 is initially present at a concentration of approximately 20  $\mu$ M to approximately 163  $\mu$ M.
- 40 64. The composition of Claim 56, wherein the inhibitor is AktI-II.

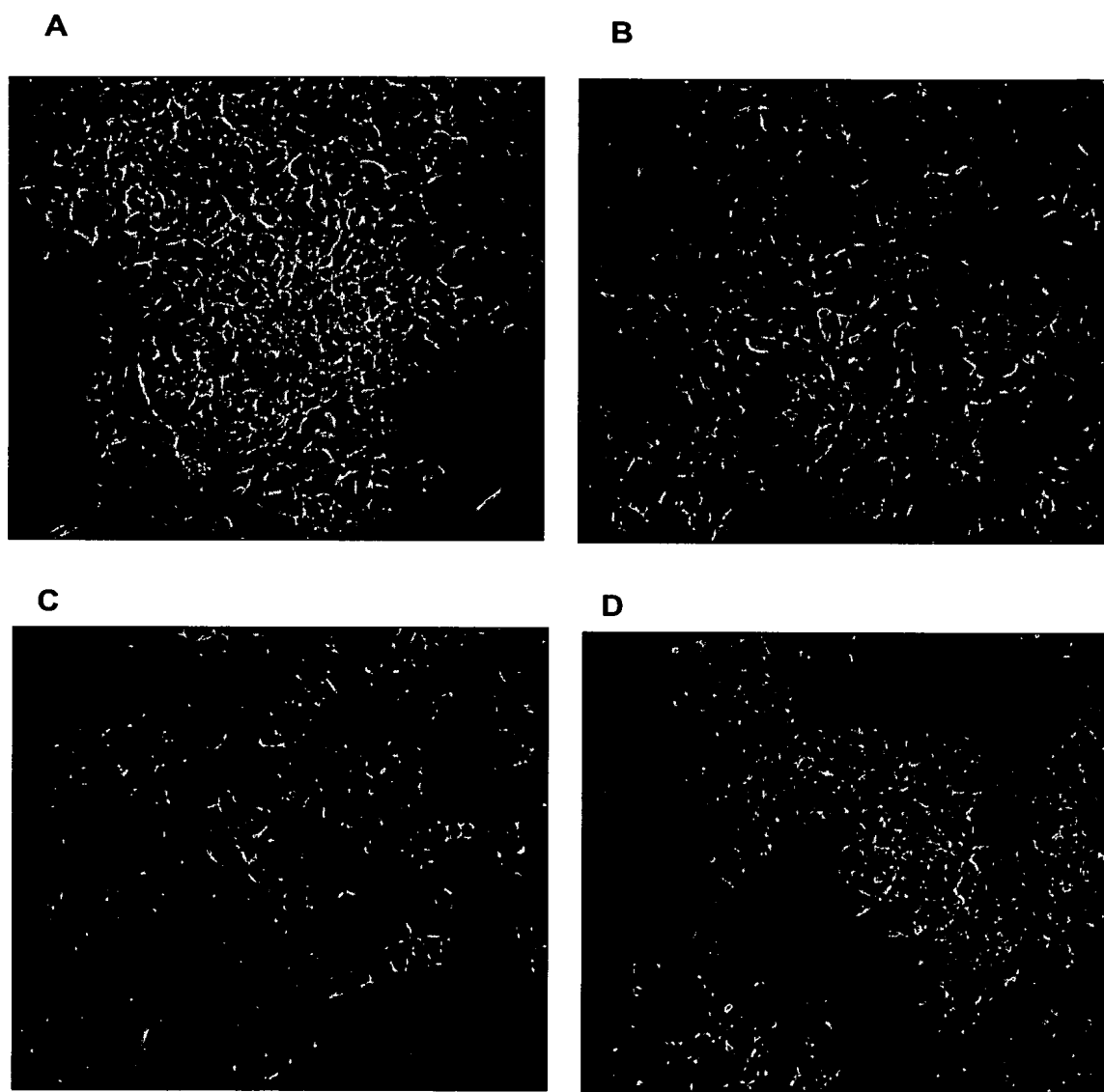
65. The composition of Claim 64, wherein AktI-II is initially present at a concentration of approximately 10  $\mu$ M to approximately 50  $\mu$ M.
66. The composition of Claim 56, wherein the member of the TGF $\beta$  family is selected from the group consisting of Nodal, Activin A, Activin B, TGF- $\beta$ , BMP2, BMP4, and mixtures of the foregoing.
67. The composition of Claim 56, wherein the member of the TGF $\beta$  family is Activin A.
68. The composition of Claim 56, wherein the member of the TGF $\beta$  family is present in a conditioned medium.
69. The composition of Claim 56, further comprising bFGF.
70. The composition of Claim 69, wherein bFGF is initially present at a concentration of approximately 1 ng/ml to approximately 12 ng/ml.
71. The composition of Claim 56, wherein the cell culture medium is a conditioned medium.
72. The composition of Claim 71, wherein the conditioned medium is obtained from a feeder layer of embryonic fibroblasts.
73. The composition of Claim 56, wherein contact with the composition is effective in causing differentiation of a pluripotent mammalian cell towards an endodermal lineage after the cell has been cultured with the composition for greater than approximately 24 hours.

**Figure 1**





**Figure 2**



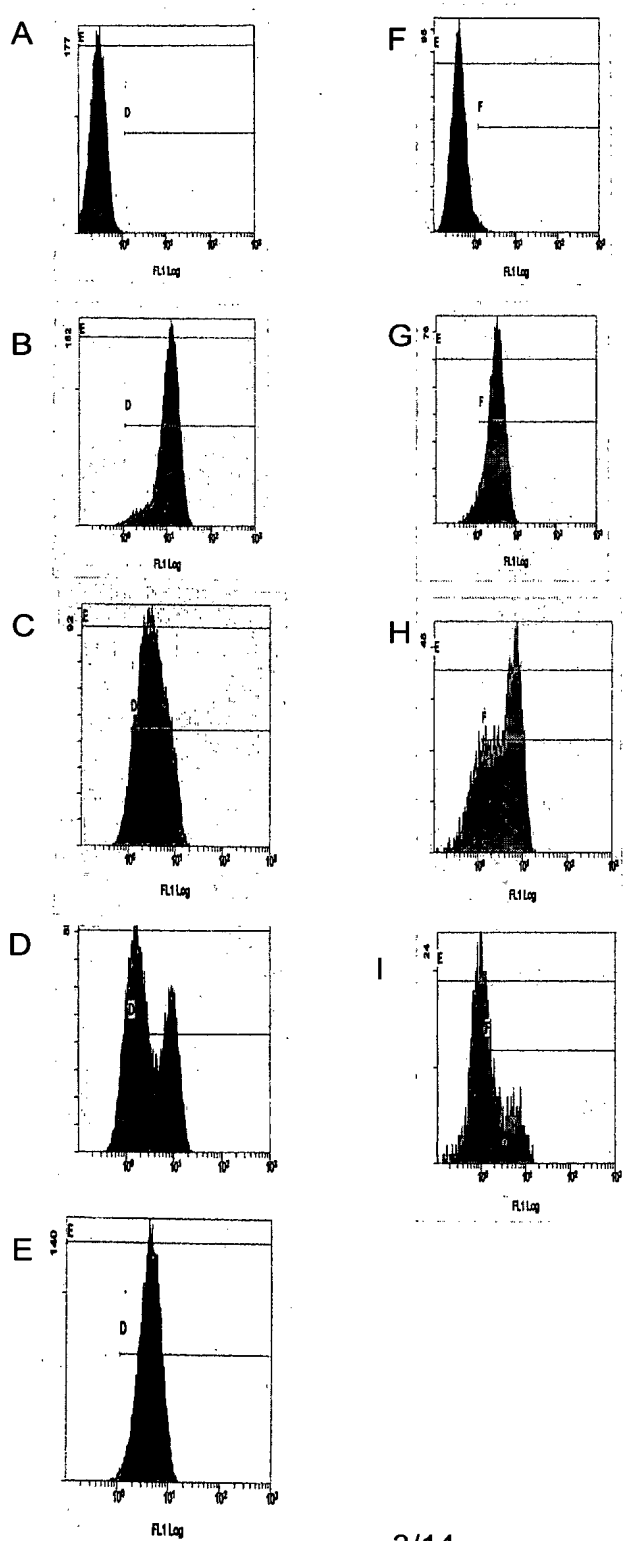
**Figure 3**

Figure 4A

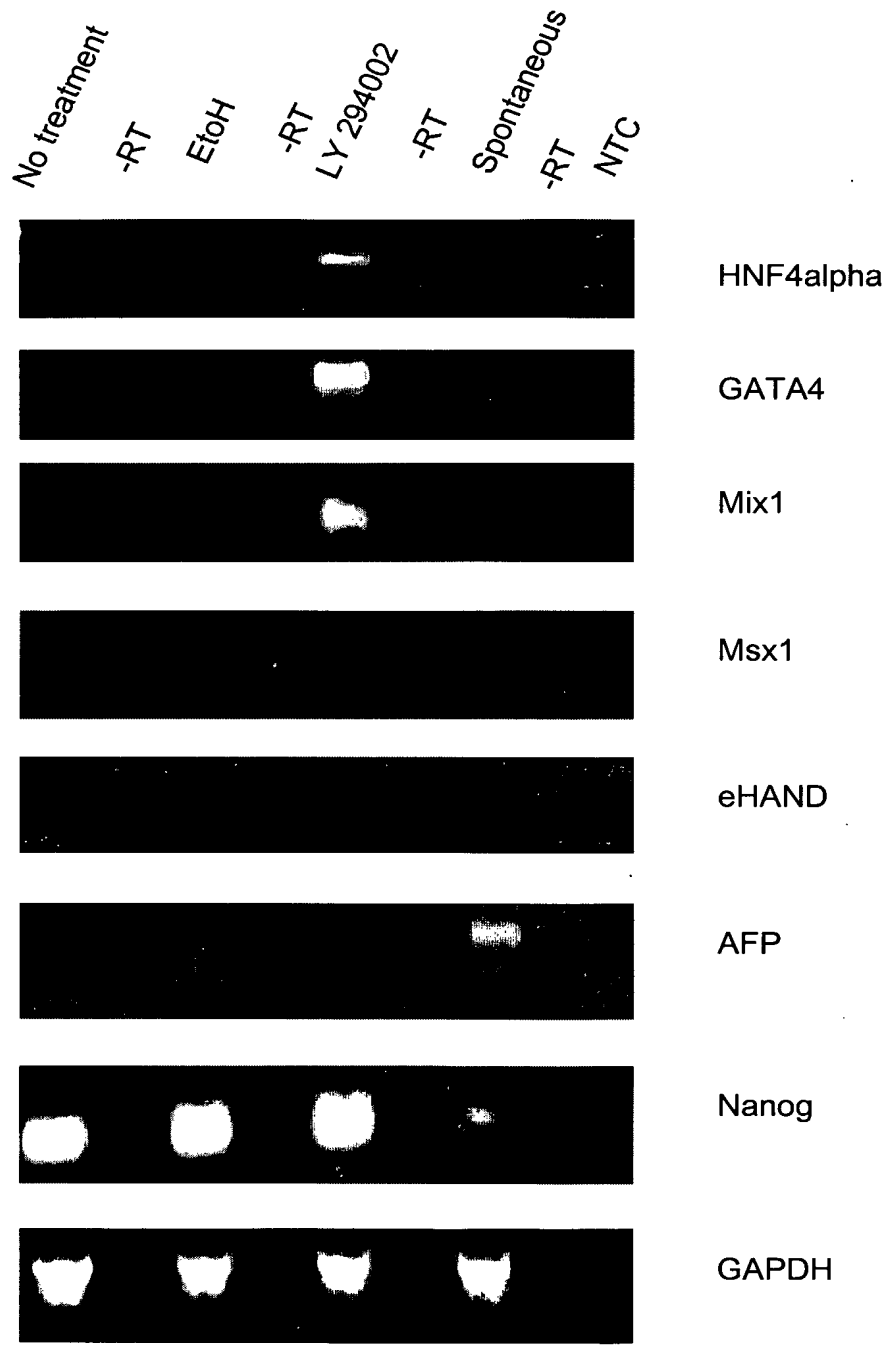
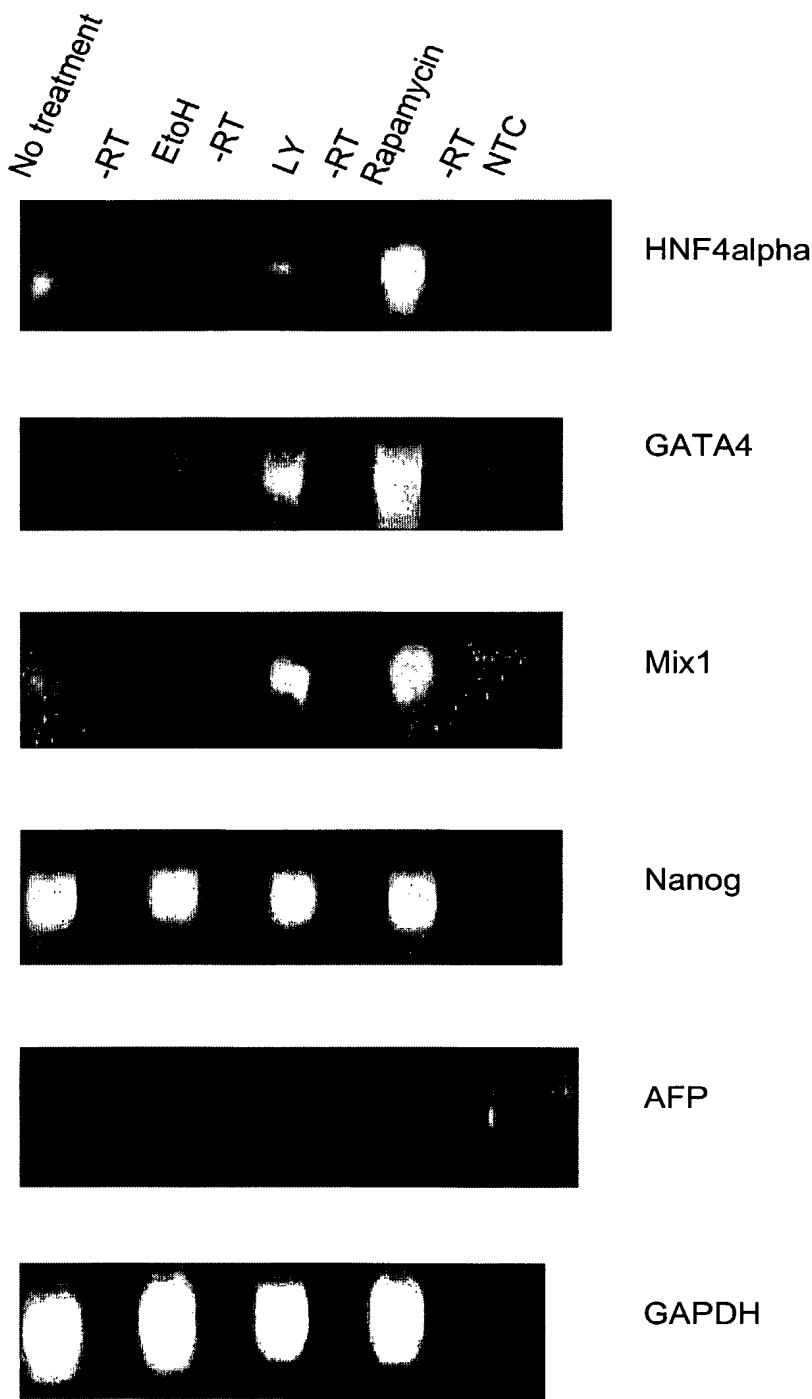
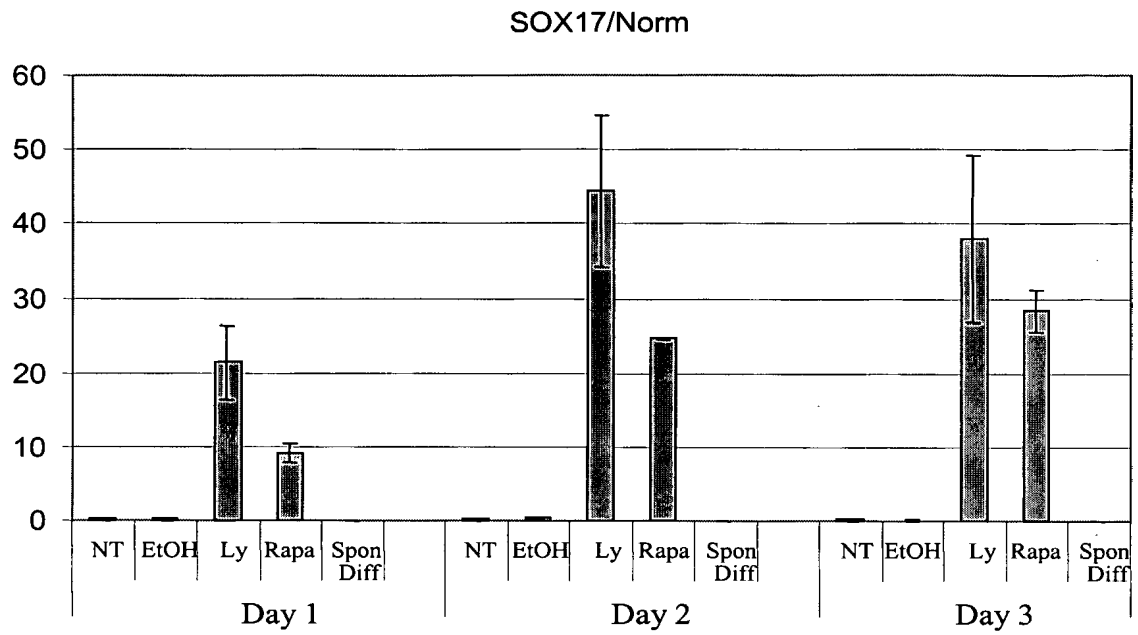
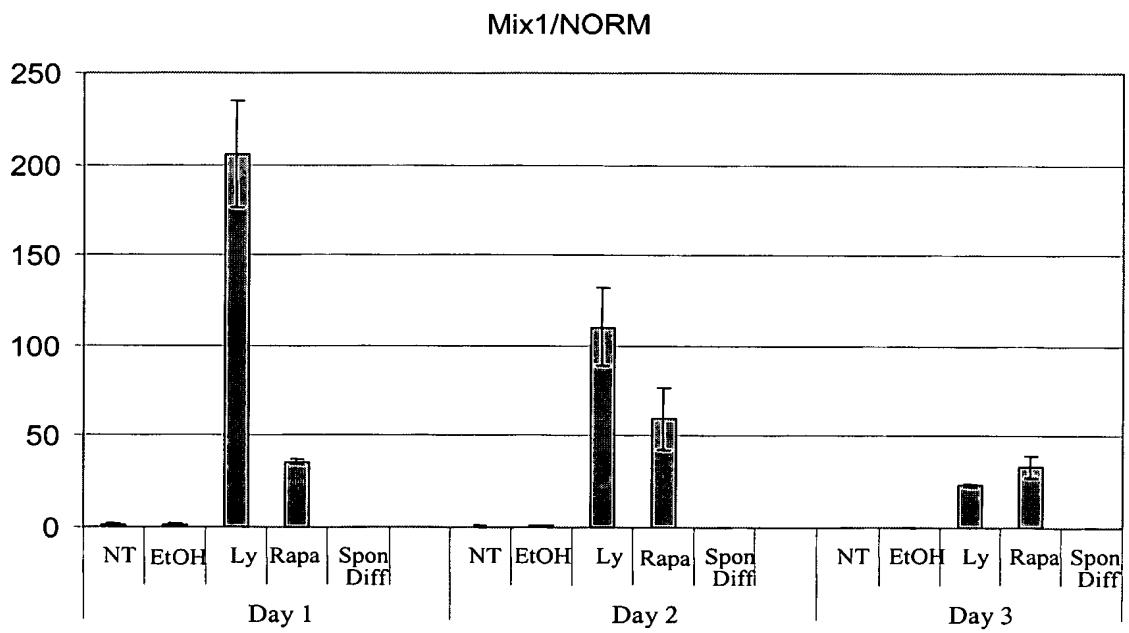
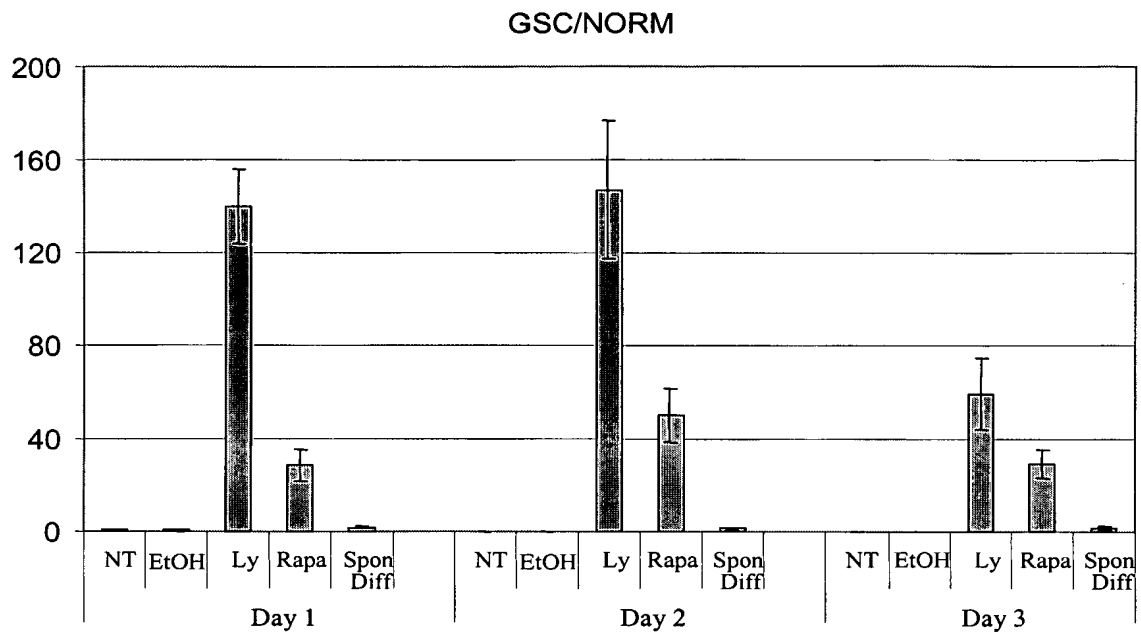
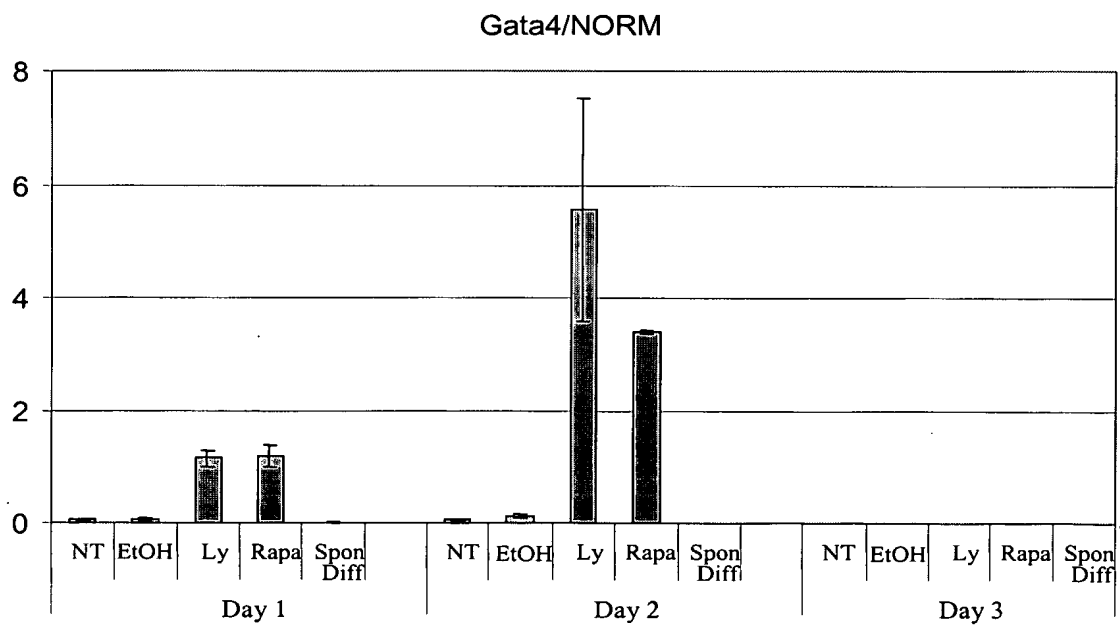


Figure 4B



**Figure 5A****Figure 5B**

**Figure 5C****Figure 5D**

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Figure 5E

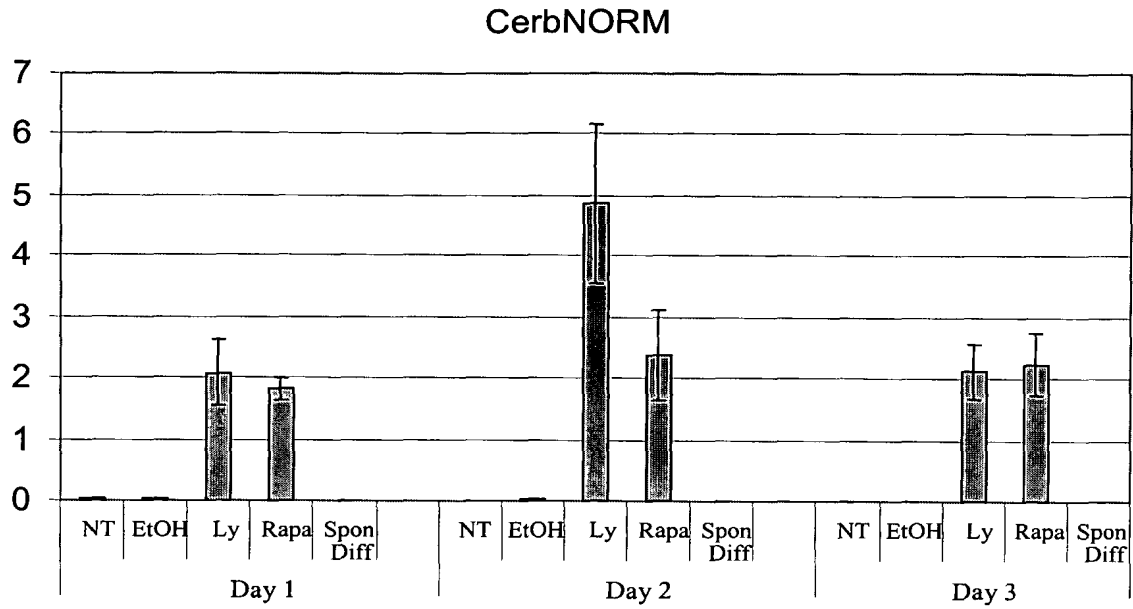
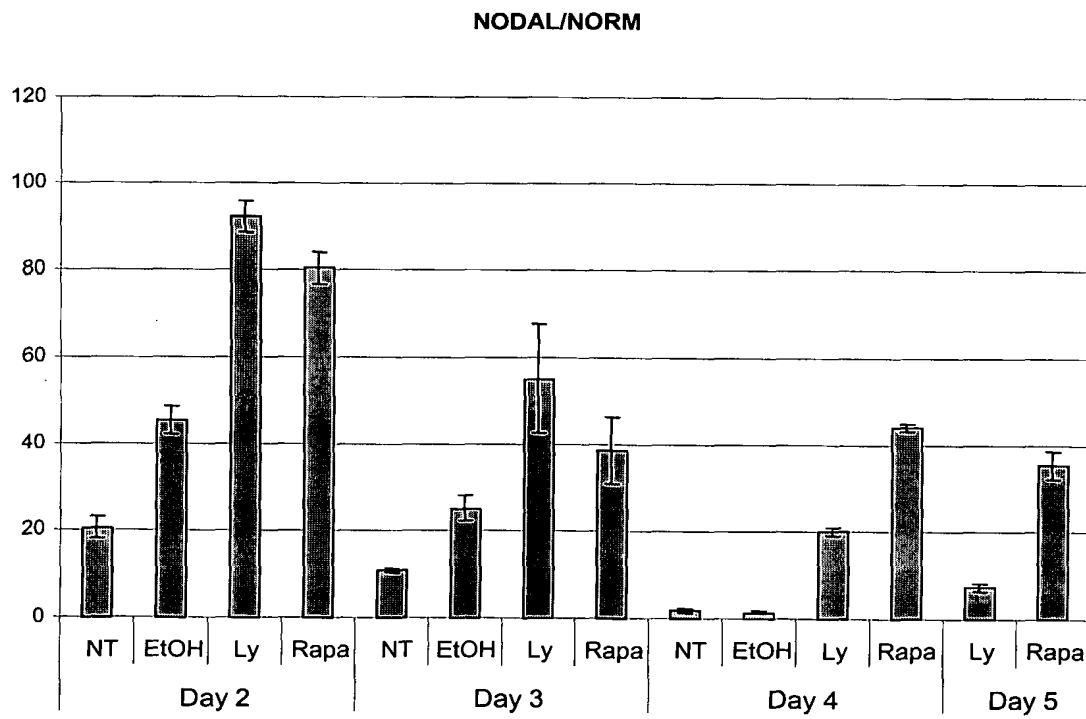
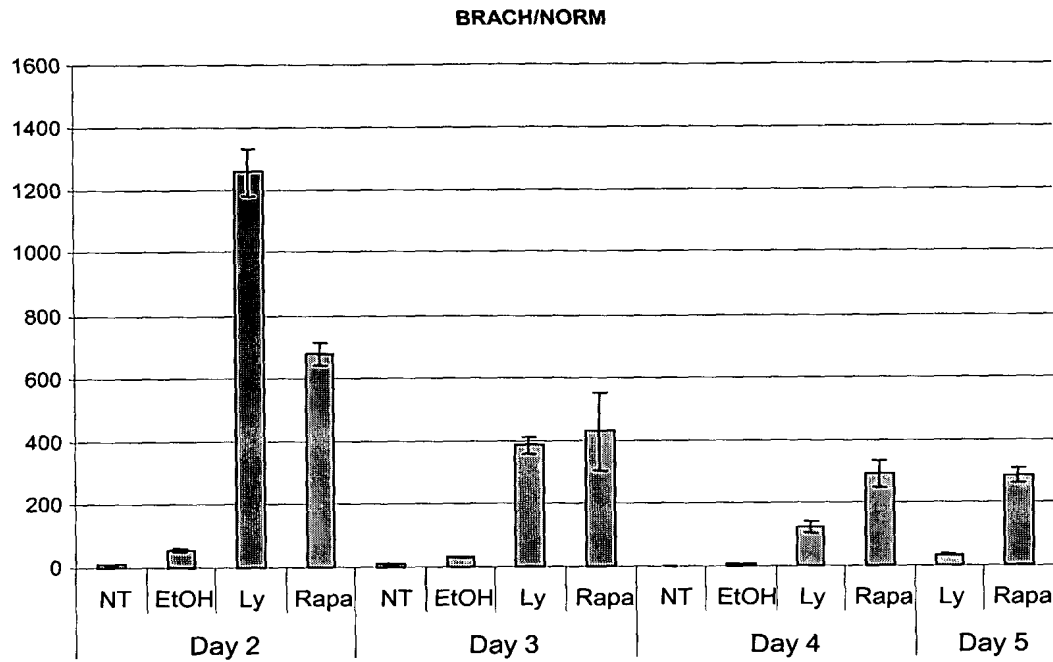
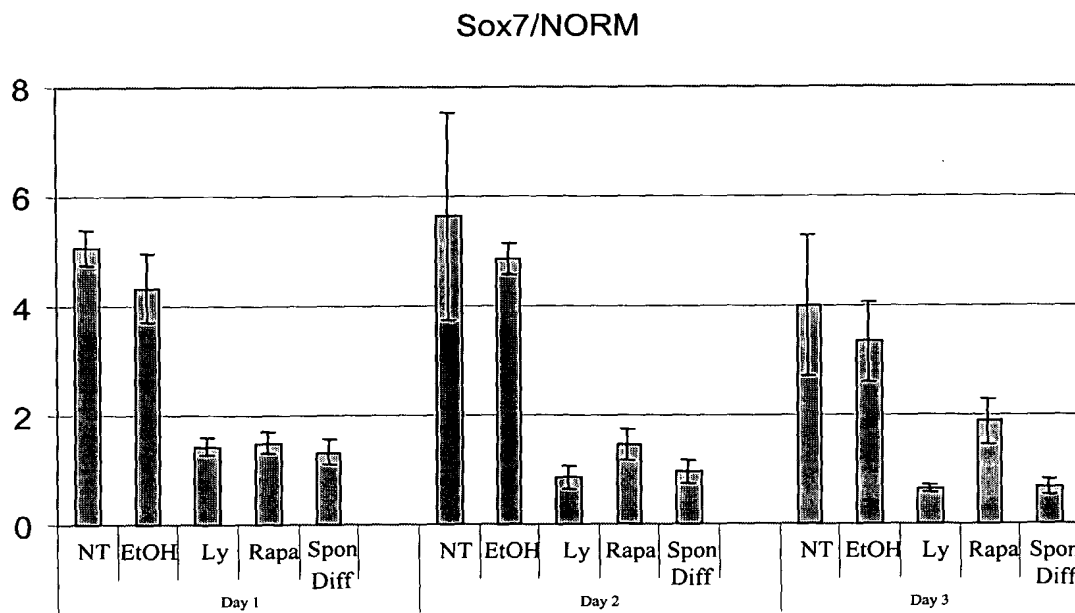


Figure 5F



**Figure 5G****Figure 5H**



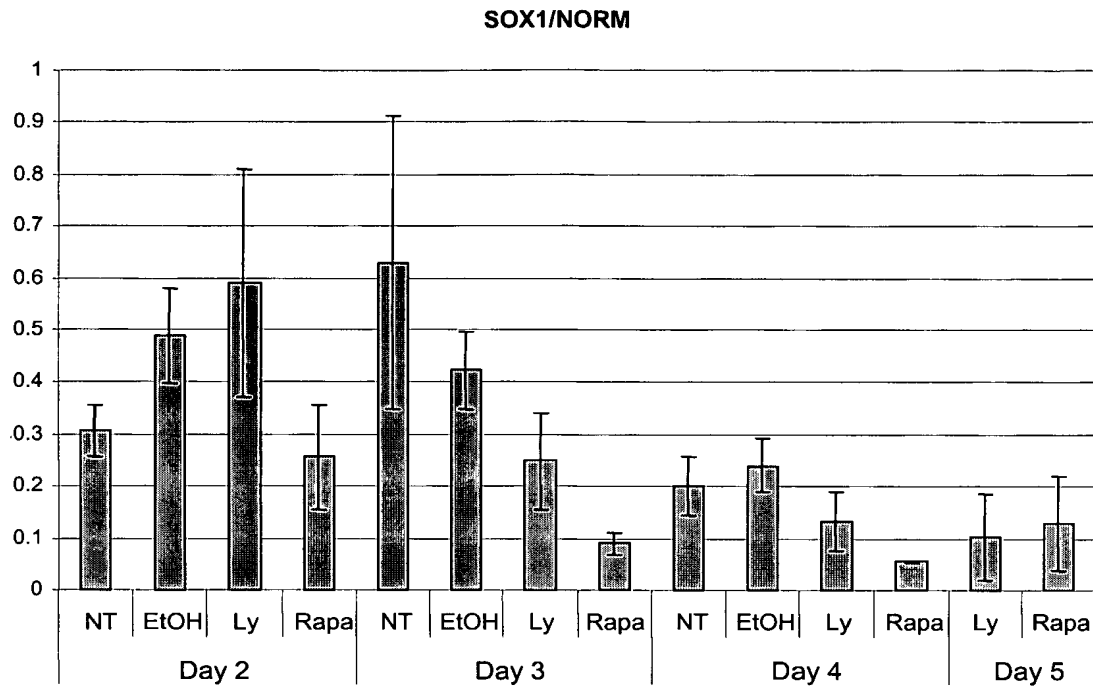
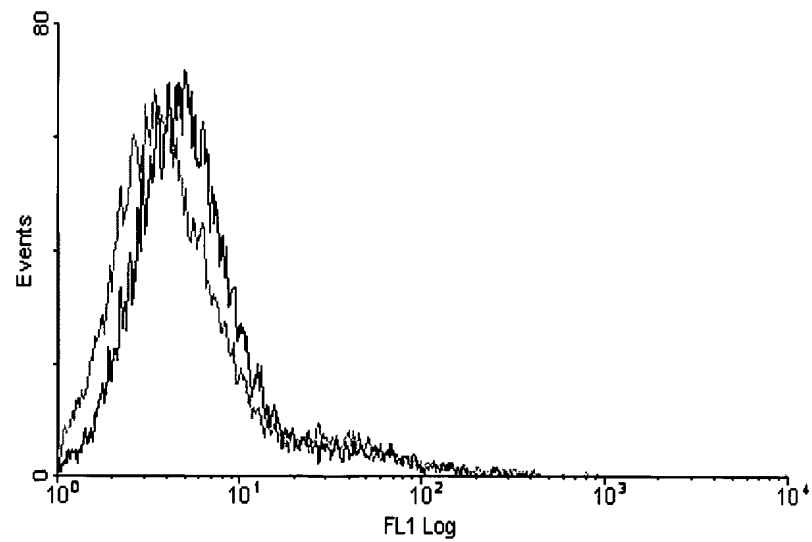
**Figure 5l****Figure 6**

Figure 7

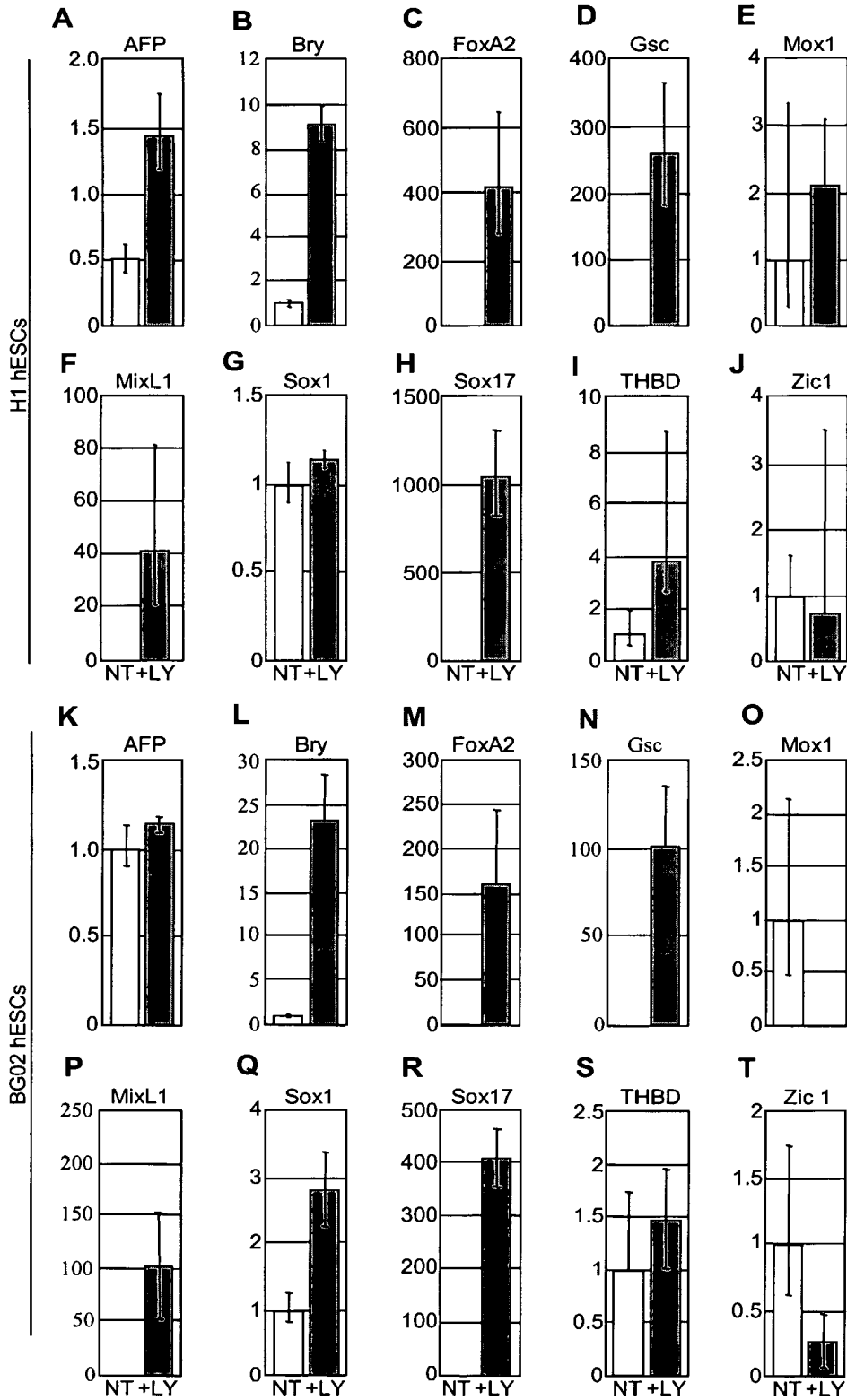


Figure 8

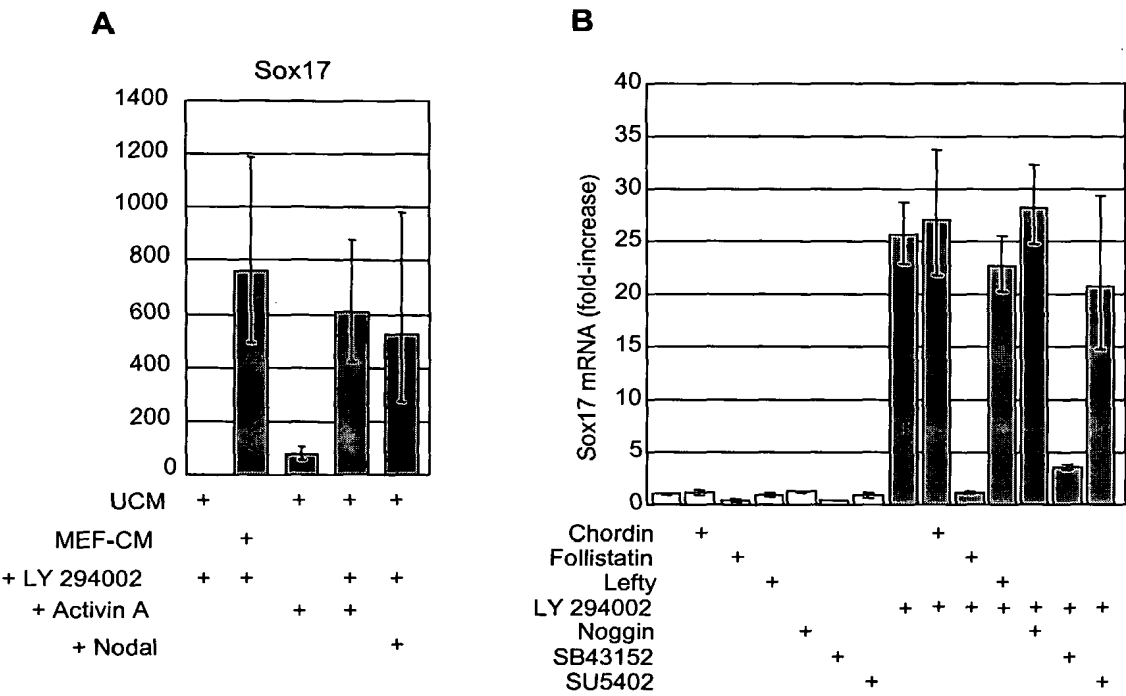
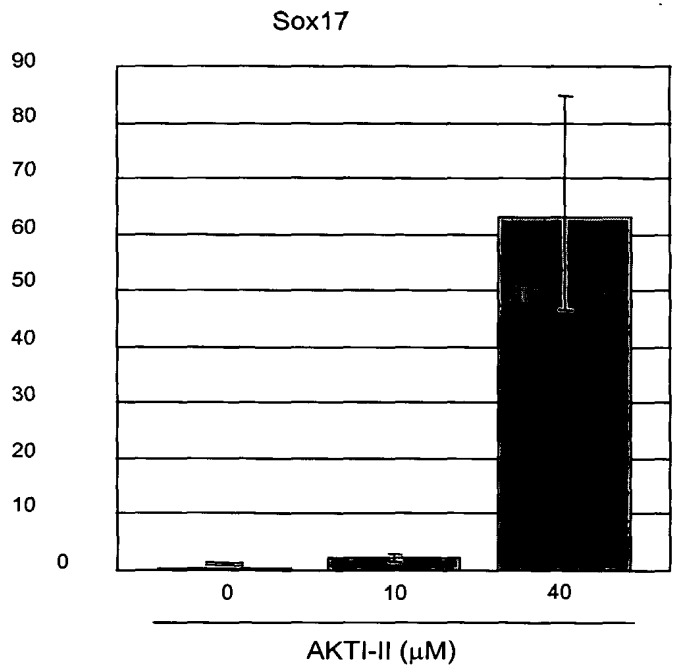


Figure 9



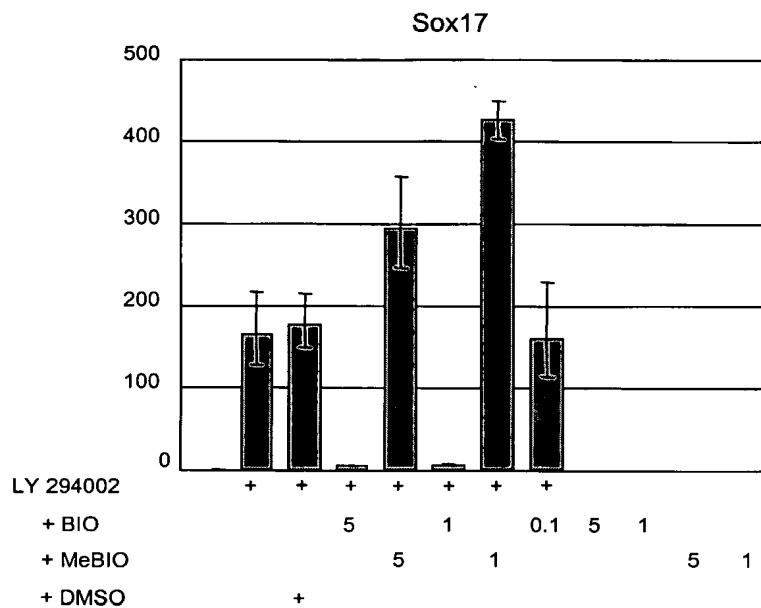
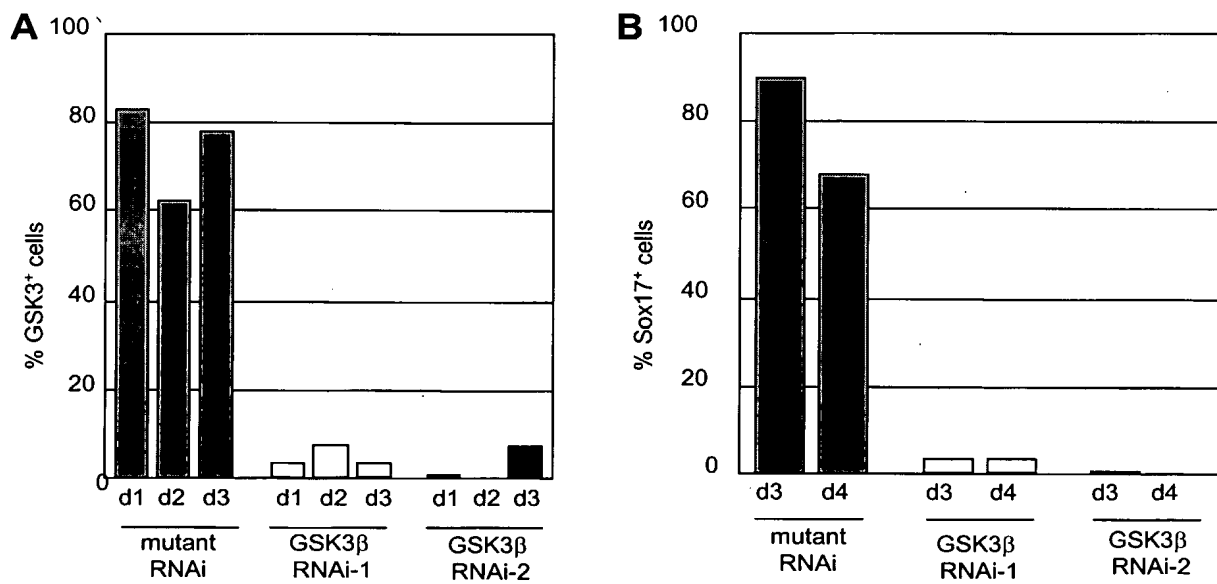
**Figure 10****Figure 11**

Figure 12

